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Title: Biocompatible material with a novel functionality

### Technical field of the invention

5 The invention is in the area of biomaterials, i.e. those materials that are used in contact with living tissue and biological fluids for prosthetic, therapeutic, storage or other applications. The working environments of any biomaterial are either biological fluids or living tissue, and the events occurring at the contacting interface play a crucial role in the overall performance of a biomaterial. Many conventional biomaterials lack the ability to properly interact with or support biological matter coming into contact with  
10 said biomaterials leading to undesired biological responses. However, these undesired responses may be controlled by altering the chemical and physical properties of the surface of said biomaterials. In this respect, surface modification represents a well known strategy of providing suitable biocompatible materials. The present invention teaches a novel approach of creating biomaterial surfaces, said surfaces being capable  
15 of functionally interacting with biological material.

### Background of the invention

When biological and synthetic materials interact with each other, one must contemplate that an association is formed that is normally not part of a biological  
20 environment such as e.g. the human or animal body. A biocompatible material has been defined as a material that, when interacting with biological material, does not induce an acute or chronic inflammatory response and does not prevent a proper differentiation of implant-surrounding tissues.<sup>[1]</sup> Furthermore, according to another current understanding, are biocompatible materials capable of i) controlling or guiding  
25 cell growth and tissue organization, ii) promoting or inhibiting cell-cell or cell-tissue interactions,<sup>[2]</sup> iii) isolating transplanted cells from the host immune system,<sup>[3]</sup> and iv) regulating production and/or secretion of cellular products.

However, many synthetic materials which are used as biomaterials are not biocompatible according to this definition, and many efforts are undertaken to find  
30 ways to improve the biocompatibility of these materials.

One important example for the interaction of biological and synthetic materials, is the adhesion of human or animal cells to polymer substrata: Cell adhesion is known to

involve various adhesive proteins, such as e.g. fibronectin (FN) and vitronectin (VN), that are adsorbed to the surface of the synthetic material and mediate a contact between said surface and adhering cells.<sup>[4,5,6,7,8]</sup> These interactions are furthermore mediated by specific transmembrane receptors belonging to the integrin family of cell adhesion molecules.<sup>[9,10]</sup> Adsorption of proteins from biological fluids onto a surface of a polymer is dependent on the physico-chemical properties of said polymer surface.<sup>[5,15]</sup> For example, it is well known that adhesive proteins adsorb abundantly onto hydrophobic polymer surfaces, but their adsorption, mainly driven by hydrophobic interactions, leads to conformational alterations and eventually to their deactivation and/or denaturation (see Fig.1).<sup>[7,12]</sup> These conformational alterations of the adhesive protein explain the reduced or eliminated interaction between said adhesive protein and a cell,<sup>[8,13]</sup> leading to reduced polymer – cell interactions.

Furthermore, conformational alterations of proteins that adsorb to the surface of a synthetic material, as e.g. the surface of an implant or of a medical device, may also give rise to increased thrombogenicity of said material or to foreign body reactions and consecutive rejection of the implantation or medical device.

Synthetic polymers are a class of materials frequently used as biomaterials with selection criteria based on their mechanical properties, stability, and capabilities of producing predefined or desired shapes and/or morphologies. However, these materials are often not biocompatible. For example, synthetic polymers in current use for the preparation of membranes with controlled permeability, e.g. polysulfones, polyesters or polypropylene, are often less than adequate for the immobilization of tissue cells because the functionality of these cells cannot, due to the above described reasons, be maintained over sufficiently long periods of time.

However, the biocompatibility of any substratum may be controlled by altering the chemical and physical properties of said substratum. Surface modification represents a well known strategy of providing suitable biocompatible materials.

Hence, polymer surfaces are e.g. modified through the addition of charged side-groups to the polymer backbone,<sup>[14]</sup> adsorption or covalent immobilization of biologically

active proteins and peptides to the polymer,<sup>[16]</sup> and the alteration of the texture or morphology of the polymeric substrate.<sup>[17, 18]</sup>

5 It is known, that surface modification is of particular interest when performed using e.g. a selective reaction initiated under mild conditions, such as e.g. photo-grafting.<sup>[19]</sup> Using a selective reaction, the shape of the substratum, including macro- or microporous structures, as well as mechanical properties, can be established and/or preserved. Examples of surface functionalizations include macroscopically homogeneous polymeric surfaces that may i) repel cells due to charge,<sup>[20]</sup> or  
10 hydrophilicity/ flexibility,<sup>[21]</sup> or surfaces to which ii) cells may adhere via e.g. conditioning of a protein on a hydrophobic surface or via attachment or operable linkage of the protein to a peptide mimicking the binding domain of an adhesive protein.<sup>[14,21]</sup>

15 Patterns of functionalization on a  $\mu\text{m}$ -scale are well suited to create patterns capable of attaching cells as well as patterns of cell-free areas.<sup>[22]</sup> Furthermore, patterns on sub- $\mu\text{m}$  scale, made up e.g. by a mixture of adhesive peptides and charged groups,<sup>[23]</sup> are also suited as supports for cell cultures. Photo-grafting in combination with photolithographic techniques<sup>[24,25]</sup> is an established way of achieving such patterns.

20 An important general class of surface modification is the attachment of macromolecules to the underlying surface. Often, these macromolecules will exhibit hydrophilic properties and thus be solvated in an biological environment, whereas the underlying surface is e.g. of hydrophobic character. The attachment of  
25 macromolecules can be achieved through the i) physical adsorption of amphiphilic macromolecules, ii) use of self-assembled monolayers (SAM),<sup>[26,27]</sup> iii) ionic binding of charged macromolecules to surface-bound countercharges, iv) grafting of either photo- or thermally-reactive macromolecules,<sup>[24,28]</sup> and, in the case where the underlying surface is polymeric, also through the, v) entanglement of said  
30 macromolecules into the polymer surface. (see also <sup>[29,30]</sup>)

It is known to coat and shield surfaces of hydrophobic basis polymers with e.g. layers or chains of (attached) hydrophilic macromolecules in order to exclude biological

material such as proteins and consequently also cells from coming into contact with said surface. In particular well known are the shielding properties of poly(ethylene glycol) (PEG), an established hydrophilic polymer. The protein repellent character of substrata coated with PEGs is accredited to a combination of several molecular mechanisms,<sup>[4]</sup> where consensus seems to be reached that the steric stabilization forces induced by the excluded volume of the attached macromolecules represents the dominating mechanism.<sup>[31,32]</sup> It has been observed, that the protein repelling character of PEG-coated substrata is dependent on their lateral density on a substratum surface,<sup>[33,34]</sup> where a correlation between amount of adsorbed protein and lateral density was observed: the higher the lateral density of attached PEGs, the lower the adsorption of proteins. According to the above, different techniques are known which describe how to prepare coated substrata, where the coats effectively shield the underlying substratum, and where said coats can also be laterally patterned.

Recently, Sofia et al.<sup>[33]</sup> characterized protein (FN, cytochrome-c, and albumin) adsorption on PEG-grafted (molecular weights (MW): 3.4, 10, and 20 kilo Dalton (kDa)) silicon surfaces over a range of grafting densities. Additionally, the grafted amount of PEG moieties could be measured with Electron Spectroscopy for Chemical Analysis (ESCA) Protein adsorption decreased for all proteins with rising PEG grafting density and was reduced by more than 95 % for the highest PEG grafting density when compared to the unmodified silicon substrate. From measurements of the thickness of the adsorbed protein layer it was deduced, that FN, having a rod like shape, adsorbs for all PEG grafting densities "lying down" with its long axis parallel and in close contact to the surface. Thus, proteins are able to penetrate the PEG-layer to effectively adsorb to the underlying surface in between grafted chains. Sofia et al. calculated that protein adsorption started to decrease for grafting densities where grafted PEG chains began to overlap, and that protein adsorption became negligible when PEG chains were, due to the grafting density, confined to approximately half of their relaxed volume. This correlation between protein adsorption and PEG chain overlap was found for all investigated PEG MWs, and the calculations were based on the assumption, that grafted PEG chains exert the same radius of gyration or spatial dimension as in their solvated state.

Contact angles (CA) are generally used to characterize the wettability of surfaces. Wettability of a surface is related to its hydrophilicity as constituted by the moieties forming that surface. It is a very sensitive technique with a probing depth of approx. 5-10 Ångströms. CA are determined by measuring the angle defined by the phase limits of the liquid phase at a three phase boundary (solid/liquid/vapor). The three phase boundary is generated by e.g. a vapor bubble in a liquid, where the bubble is captivated by the test surface (captive-bubble method, see also Fig.2) or a drop of liquid in vapor placed on top of the test surface (sessile-drop method). When a polar (hydrophilic) test liquid (as e.g. water) is used, hydrophilic test surfaces will generate small angle values such as  $0^{\circ}$ - $90^{\circ}$ , while hydrophobic test surfaces will generate large angle values of  $90^{\circ}$ - $180^{\circ}$ . Both advancing and receding measurements of CA's are used to characterize the biomaterials according to the invention.<sup>[35]</sup>

While it is not intended that the present invention be limited by the nature of the particular mechanism or the understanding of the particular physical forces involved, a drop of liquid resting on a substratum may be considered to be balancing three forces: a) the interfacial tension between the solid and liquid, b) the interfacial tension between the solid and vapor, and c) the interfacial tension between the liquid and the vapor. The angle within the liquid phase is known as the "contact angle". See B. C. Nayar and A. W. Adamson, "Contact Angle in Industry" Science Reporter, pp. 76-79 (February 1981). It is the angle included between the tangent line to the surface of the liquid and the tangent plane to the surface of the solid at a point along their line of contact.

Advancing and receding contact angles are frequently found not to be the same. This hysteresis may be due to rough surfaces or to chemical heterogeneities of the substratum.

One method of measuring the contact angle is by taking a photograph of a bubble captivated by the substratum and then measuring the angle from the print. The angle can also be measured from an enlarged image of the bubble. A low power microscope produces a sharply defined image of the liquid bubble which is observed through the eyepiece as a silhouette.

There are commercially available goniometers with environmental chambers in which contact angles can be determined in controlled conditions of temperature and pressure. A camera can also be attached to such goniometers. Commercial instruments for measuring contact angles are available from such companies as RAME-HART, Inc. (Mountain Lakes, N.J.), KRUSS (Charlotte, N.C.), CAHN INSTRUMENTS (Cerritos, Calif.), and KERNCO INSTRUMENTS (El Paso, Tex.).

Contact angles ranges from zero (0) to one hundred and eighty (180) degrees (although the latter is not encountered in practice). In this invention, water is used as a probing liquid (representing the liquid phase), and air saturated by water vapor as the gas phase (forming the bubble). When the contact angle is between approximately zero and approximately ninety (90) degrees, the substratum is considered hydrophilic. Ninety (90) degrees is considered "hydroneutral." When the contact angle is greater than ninety (90) degrees, the substratum is considered hydrophobic.

Ellipsometry is an optical in situ technique for measuring i) the refractive index of a bare surface, or ii) the thickness and refractive index of a film/coat on a substratum, both based on measuring the change in the state of polarization of light upon reflection from said substratum surface. The determined thickness and refractive index of an adsorbed layer of macromolecules can thus be converted to a value of adsorbed mass.[36] In this way it is possible to monitor on-line the adsorption of e.g. macromolecules out of solution onto a substratum surface interfacing that solution. There are detailed descriptions of the physical principles of the method[37] and the instrumental setup.[38]

### **Studying cell adhesion**

The quality of the interactions of a synthetic material surface with biological material, i.e. the biocompatibility of said material surface, can be related to the behavior of living cells when in contact with said surface. Accordingly, criteria like the amount of adhered cells, overall cell morphology, cell migration, focal adhesion formation, extra cellular matrix (ECM) formation, and cell proliferation on the material surface are

considered important when aiming to monitor and control the biocompatibility of a material surface in vitro.

5 The below-identified documents are believed to constitute the closest prior art in relation to the present invention. The prior art teaches how to shield a polymer from biological material, it does not address the technical problem solved by the present invention.

10 **Sofia et al. (1998), Macromolecules 31, 5059-5070**, compares different PEG type molecules, and their interaction with proteins when chemically grafted to a polymer substratum. The document does not disclose the binding of biological material in an active form to the disclosed polymer material.

15 **US patent no. 5,776,748** is related to a device comprising a plurality of cytophilic islands and cytophobic regions established by self-assembled monolayers exhibiting cytophilic or cytophobic endgroups. Cell-adhesion is promoted or inhibited on the cytophilic or cytophobic regions respectively by known mechanism, as e.g. introduction of polar groups, charges, and others, and does not disclose the binding of biological material in an active form.

20 **US patent no. 5,002,582** is related to a method of producing biomaterials having an "effective" solid surface characterized by the properties of the hydrophilic polymer and not of the solid hydrophobic surface (column 8). The claimed biomaterials do not have a contact angle that is substantially similar to that of the solid surface. The document does not disclose the binding of biological material in an active form to the disclosed polymer material.

25 **US patent no. 4,973,493** is related to a method of producing a solid surface that is effectively shielded by a biocompatible agent. The claimed biomaterials are unlikely to have a contact angle that is substantially similar to that of the solid surface. The document does not disclose the binding of biological material in an active form to the disclosed polymer material.

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US patent no. 4,722,906 is related to a method for selectively binding specific molecular target moieties covalently to a chemical moiety or substratum. The document does not disclose the binding of biological material in an active form to the disclosed polymer material.

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US patent no. 5,128,170 is related to a method for manufacturing a medical device having a highly biocompatible surface. The claimed biocompatible surface does not have a contact angle that is substantially similar to that of the medical device. The document does not disclose the binding of biological material in an active form to the disclosed polymer material.

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US patent no. 5,728,437 is related to an article comprising a hydrophobic surface coated with a blood compatible surface layer. The coated surface does not have a contact angle that is substantially similar to that of the hydrophobic surface. The document does not disclose the binding of biological material in an active form to the disclosed polymer material.

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US patent no. 5,380,904 is related to a method for rendering a surface biocompatible. The biocompatible surface does not have a contact angle that is substantially similar to that of the untreated surface. The document does not disclose the binding of biological material in an active form to the disclosed polymer material.

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US patent no. 5,512,329 is related to methods of attaching a polymer to a surface of a substrate by application of an external stimulus. The method of claim 14 is directed to a method of modifying surface properties of a substrate. A biomaterial comprising a polymer substratum and a macromolecule and a first determinant capable of bringing a second determinant into contact with said first determinant is not disclosed. Neither does the document disclose the binding of biological material in an active form to the disclosed polymer material.

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US patent no. 5,217,492 is related to a specialized means for attaching a biomolecule to a hydrophobic surface. The disclosed means for attachment is not pertinent to the



present invention. The document does not disclose the binding of biological material in an active form to the disclosed polymer material.

5        **US patent no. 5,263,992** is related to a biocompatible device comprising a solid surface and a biocompatible agent positioned sufficiently proximate to one another so as to effectively shield the solid surface. The document does not disclose the binding of biological material in an active form to the disclosed polymer material.

10       **US patent no. 5,741,881** is related to a bio-active coating that exploits a hydrophilic spacer with functional end groups and capable of linking a specialized polymer with a bio-active agent. The present invention does not exploit a bifunctional linker in the form of a hydrophilic spacer as a means for attaching a first determinant to a polymer substratum. The document does not disclose the binding of biological material in an active form to the disclosed polymer material.

15       **WO 97/46590** is related to a material comprising a support and two layers, of which the second, outer layer is a hydrophilic polymer, said material further comprising immobilized biological material. The surface generated by coating a support with a polymeric surfactant and hydrophilic polymer does not have a contact angle that is  
20       substantially similar to that of the support. The document does not disclose the binding of biological material in an active form to the disclosed polymer material.

25       **WO 97/18904** is related to a method for providing a hydrophobic surface with a hydrophilic coating. The surface generated by hydrophilic coating does not have a contact angle that is substantially similar to that of the hydrophobic surface. The document does not disclose the binding of biological material in an active form to the disclosed polymer material.

30       **EP 633 031 A1** is related to a composition that is effectively capable of shielding a polymer from biological material. The shielded polymer does not have a contact angle that is substantially similar to that of the unshielded polymer. The document does not disclose the binding of biological material in an active form to the disclosed polymer material.

**Park and Griffith (1998), J. Biomat. Sci. Polym. Ed. 9, p. 89-110**, discloses a specialized PEG-PPO-PEG copolymer scaffold capable of effectively inhibiting cell adhesion. The copolymer is useful in regulating the three dimensional organization of diverse cell types. Adhesion is achieved by covalent linkage to the polymer of a cell specific carbohydrate ligand capable of binding a particular receptor moiety. The present invention is not concerned with a polymer substratum being contacted with a first determinant. The cell adhesive properties of the biomaterial according to the present invention are at least partly determined by the cooperativity of a polymer substratum and a macromolecule and optionally also by a first determinant. The polymer "backbone" of the present invention is not cytophobic per se, as is the case in the cited reference. The disclosed copolymer material is not pertinent to the present invention, and the document does not disclose the binding of biological material in an active form to the disclosed polymer material.

**Noh et al. (1998), J. Biomat. Sci. Polym. Ed. 9, p. 407-426**, discloses a modification of PTFE films that substantially alters the contact angle. The disclosed biomaterials are not pertinent to the present invention, and the document does not disclose the binding of biological material in an active form to the disclosed polymer material.

**Malmsten et al. (1998), J. Coll. and Interface Science 202, p. 507-517**, examines the effect of chain density on inhibition of protein adsorption. The document does not mention the properties of the bound proteins, and the document does not disclose the binding of biological material in an active form to the disclosed polymer material.

**Zhang et al. (1998), Biomaterials 19, p. 953-960**, discloses silicon surfaces that are modified with a PEG film in order to reduce protein adsorption. The silicon surface does not have a contact angle that is substantially similar to that of the PEG-coated material. The document does not disclose the binding of biological material in an active form to the disclosed polymer material.

**Herbert et al. (1997), Chemistry and Biology 4, p. 731-737**, discloses a method of differentiating the cross-linking of bioactive molecules to a surface. Biomaterials

according to the present invention are not disclosed and the disclosed method is not pertinent to the present invention as photo-reactivation is acknowledged to form part of the prior art. The document does not disclose the binding of biological material in an active form to the disclosed polymer material.

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**Wesslén et al. (1994), *Biomaterials* 15, p. 278-284**, discloses a surface modification of a hydrophobic polymer by use of hydrophilic polymers including PEG. The modification significantly changes the contact angles (Table 1) and leads to a reduced polypeptide adhesion. The disclosed biomaterials are not pertinent to the present invention, and the document does not disclose the binding of biological material in an active form to the disclosed polymer material.

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**Bergström et al. (1992), *J. Biomedical Materials Research* 26, p. 779-790**, discloses a polystyrene comprising densely packed and covalently bound PEG capable of effectively reducing adsorption of fibrinogen. The polystyrene does not have a contact angle that is substantially similar to that of the densely packed PEG surface. The document does not disclose the binding of biological material in an active form to the disclosed polymer material.

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**Desai and Hubbell (1991), *Biomaterials* 12, p. 144-153**, discloses an incorporation of PEG and similar water-soluble polymers onto surfaces of biomedical polymers such as e.g. PET and the like. The incorporation significantly alters the contact angle as illustrated in Table 1. The disclosed biomaterials are not pertinent to the present invention, and the document does not disclose the binding of biological material in an active form to the disclosed polymer material.

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**Gombotz et al. (1991), *J. Biomedical Materials Research*, 25, p. 1547-1562**, discloses a modification of PET surfaces with PEG. The incorporation significantly alters the contact angle as illustrated in and the first paragraph of the discussion. The disclosed biomaterials are not pertinent to the present invention, and the document does not disclose the binding of biological material in an active form to the disclosed polymer material.

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**Summary of the invention**

The present invention teaches a novel approach of creating biocompatible surfaces, said surfaces being capable of functionally interact with biological material. Said biocompatible surfaces comprise at least two components, such as a hydrophobic substratum and a macromolecule of hydrophilic nature, which, in a cooperativity, form together the novel biocompatible surfaces.

The novel approach is based on contacting said hydrophobic substratum with a laterally patterned monomolecular layer of said hydrophilic and flexible macromolecules, exhibiting a pronounced excluded volume. The thus formed two component surface is, in respect to polarity and morphology, a molecularly heterogeneous surface. Structural features of said macromolecular monolayer (as e.g. the layer thickness or its lateral density) are determined by, i) the structural features of the layer forming macromolecules (as e.g. their MW or their molecular architecture) and, ii) the method of creating said monomolecular layer (as e.g. by physis- or chemisorbing, or by chemically binding said macromolecules). The structural features of the layer forming macromolecule(s) is in turn determined by synthesis.

Amount and conformation and thus also biological activity of biological material (as e.g. polypeptides) which contact the novel biocompatible surface, is determined and maintained by the cooperative action of the underlying hydrophobic substratum and the macromolecular layer. In this way it becomes possible to maintain and control biological interactions between said contacted polypeptides and other biological compounds as e.g. cells, antibodies and the like. Consequently, the present invention aims to reduce and/or eliminate the deactivation and/or denaturation associated with the contacting of polypeptides and/or other biological material to a hydrophobic substratum surface.

In a preferred hypothesis, solvated polypeptides penetrate the laterally patterned monolayer of macromolecules to effectively adsorb in-between said macromolecules to the underlying hydrophobic surface. Said polypeptides must, in order to penetrate the monolayer of macromolecules, deform said self-assembled macromolecules to some degree, inducing a lateral pressure acting between said macromolecules and

penetrated polypeptides, but also between said macromolecules themselves (see also Fig.3). This lateral pressure has its origin in the unfavorable loss in conformational entropy of said bound macromolecules related to the spatial deformation of said macromolecules. The lateral pressure will therefore increase as the amount of penetrated polypeptides increases.

Consequently, the amount of adsorbed polypeptides will, according to the hypothesis, continue to increase until an energetically favorable balance is attained between, i) the unfavorable induced lateral pressure, and ii) the favorable adsorption of said polypeptides to the underlying hydrophobic surface. Polypeptides will therefore continue to penetrate the macromolecular layer to effectively adsorb to the underlying hydrophobic surface until the hereby induced lateral pressure in that layer will effectively repel any other polypeptides from that layer.

According to this hypothesis, polypeptides adsorbed in-between said self-assembled macromolecular layer will be exposed to a lateral pressure originating from surrounding and deformed macromolecules. The lateral pressure acting upon adsorbed polypeptides, will effectively protect said polypeptides from unfolding/denaturation, and stabilize said polypeptides in an active conformation, yielding adsorbed but biologically active polypeptides.

The invention thus solves the problem of how to provide - by simple and inexpensive methods - general surface design principles and modification methods in order to enable e.g. the control of attachment, spreading, growth and tissue formation of cells on surfaces, as these depend on biologically active polypeptides present at a surface. These novel biocompatible surfaces may thus be used as cell-culture dishes, bioreactors, implants, biohybrid organs such as pacemakers, and the like, without the need of extensive development of new polymers and biocompatibility screening.

It is therefore contemplated, that the present invention provides means to create biocompatible surfaces suitable for use in emerging technologies such as e.g. the construction and application of novel surface architectures of biomaterials with innovative functionalities. Accordingly, the invention is useful in the manufacture of

surface architectures for use in biohybrid organs, such as e.g. a bioartificial pancreas, liver or kidney. The invention will enable the use of improved membranes for ensuring spatial separation of e.g. xenogenic and/or allogenic cells from the host immune system.

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Modifying membranes with said macromolecular layers comprising hydrophilic macromolecules such as e.g. PEG may according to the present invention reduce the amount of adsorption of proteins on the plane of the membrane<sup>[24]</sup> and at the same time improve the conformational/functional state/form of adsorbed proteins such as FN and other attachment proteins.

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The present invention also contemplates providing arrays for culturing "sensual" cells such as e.g. nerve, olfactorial, retina, and similar cells. Culturing of sensual cells requires a spatially resolved reception of signals that must be organized in a highly complex and specific manner. The signals generated by those cells must be transmitted to a non-biological support in a time resolved and location dependent manner. Photolithographical techniques involving e.g. the immobilization of PEG spacers and bio-specific ligands may be used to contribute to the structuring and/or functionalization of solid supports in a highly specific way. It is envisaged that such structures may eventually be used e.g. as sensors or biohybrid organs.

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Cells capable of being immobilized onto the biomaterials according to the invention are preferably, but not limited to, cells the function of which comprise i) controlled delivery of biologically active substances, such as e.g. hormones, ii) production of predetermined proteins and polypeptides derivable therefrom, such as e.g. antibodies, growth factors, matrix factors, and the like, or iii) the conversion of metabolites, preferably toxic or cytostatic metabolites. Examples for such types of cells are e.g. Langerhans islets cells, hybridoma cells, chondrocytes, and hepatocytes.

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It is contemplated that the invention is useful in the organization of cells in organs and tissues. Such an organization involves a controlled co-operation of different types of cells that are connected, on a micrometer scale, through a local and highly organized network of different cell types. It is contemplated that the present invention will allow

photolithographical techniques to be applied in the immobilization of macromolecules with distinct functionalities and biogenic ligands. The biomaterials thus generated are capable of immobilizing different types of cells in a controlled and/or spatially structured manner so as to make them available for a controlled co-operation.

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It is also contemplated to obtain an organization of cells in organs and tissue-like structures by stochastically distributed macromolecules (e.g. with and without specific functionalities, such as, e.g. amine groups, either itself or for subsequent immobilization of biological or biomimetic receptors) on a solid support, and subsequently use a second ligand (e.g. another macromolecules with a different functionality such as e.g. a functionality exerted by e.g. a different chain length) in the formation of clusters of different sizes (e.g. clusters with a different length with regard to an axis, e.g. the z-axis) and/or functionality. In this way, the invention makes it possible to obtain a patterning of a given substrate in three dimensions. This may eventually offer the possibility of providing structured surfaces for the immobilization of e.g. a single type of cells, or e.g. co-culture different cells by binding ligands that are selective for specific cell surface receptors, such as integrins, growth factor receptors and the like.

The novel and innovative applications described herein above cannot be realized with the state of the art means currently available, because there exists a profound lack of useful design principles and suitable methods for surface modification. Also, the state of the art methods are not readily applicable to fine-tune the surface structure and/or biocompatibility of known polymeric biomaterials. The invention described herein represents a significant improvement of the state of the art techniques and potentially enables the creation of novel biocompatible materials and cell-based technologies.

According to one preferred aspect of the present invention, the biocompatible material surface has a contact angle that is substantially identical to the contact angle of the underlying hydrophobic substratum of said surface. Substantially identical contact angles within the meaning of the present invention will be understood to comprise any change of contact angle within the numerical value of less than 5 degrees, such as less than 4.5 degrees, for example less than 4.0 degrees, such as less than 3.7 degrees, for

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example less than 3.3 degrees, such as less than 3.0 degrees, for example less than 2.8 degrees, such as less than 2.5 degrees.

5 The biocompatible surface according to the invention differs from prior art hydrophobic substrata that are coated with a hydrophilic layer, as such prior art surfaces have a contact angle that is significantly different from that of the basis substratum. Consequently, the invention relates to conversion of a hydrophobic substratum having a predetermined contact angle into a biocompatible material surface having essentially the same contact angle but having another functionality  
10 with respect to biologically active moities, such as polypeptides, proteins, cells, etc. being in contact with said substratum. The biocompatible surface may further comprise a first determinant, e.g. an adhesion polypeptide, capable of bringing a second determinant, e.g. a biological cell, into reactive contact with said first determinant.

15 In yet another aspect of the invention, the biocompatible surface is capable of interacting with at least one first determinant (e.g. a polypeptide) and maintain said first determinant in an active form, preferably an active conformation. The presence of said first determinant in its functional form and/or active conformation results in an  
20 improved first determinant-mediated contact between said biocompatible surface comprising said first determinant and e.g. a second determinant such as a cell capable of contacting said first determinant and preferably forming a stable association therewith.

25 The first and second determinant may in one embodiment independently of one another comprise a cell or consist of a cell. The cell is preferably selected from the group consisting of adipocytes, astrocytes, cardiac muscle cells, chondrocytes, endothelial cells, epithelial cells, fibroblasts, gangliocytes, glandular cells, glial cells, hematopoietic cells, hepatocytes, keratinocytes, myoblasts, neural cells, osteoblasts,  
30 pancreatic beta cells, renal cells, smooth muscle cells, striated muscle cells, and precursors of any of the above. Additionally preferred cells are stromal tissue cells found in loose connective tissue or bone marrow, and preferably endothelial cells,



pericytes, macrophages, monocytes, leukocytes, plasma cells, mast cells, including any precursor thereof.

5 When the second determinant is a cell, preferably any one or more of the ones listed herein immediately above, the first determinant preferably comprises a polypeptide or another biological entity capable of optimising or stabilising the association formed between the material according to the invention and the cells in question.

10 In a first aspect the present invention pertains to a material comprising a substratum, said substratum being contactable with a macromolecule, said material further comprising at least one macromolecule,

said material having a first contact angle  $a$ ,

15 said substratum having a second contact angle  $b_0$  when not contacted by a macromolecule,

said contact angle  $a$  being substantially identical to said contact angle  $b_0$ .

20 In one embodiment of this aspect there is provided a material comprising a substratum, said substratum being contactable with a macromolecule, said material further comprising at least one macromolecule,

said material having a first contact angle  $a$ ,

25 said substratum having a second contact angle  $b_0$  when not contacted by a macromolecule, and another second contact angle  $b_{sat}$ , when said substratum is saturated by said macromolecules as defined herein,

30 wherein the relation between said contact angles is as defined by the ratio  $R$ ,

$$R = (b_0 - a) / (b_0 - b_{sat})$$

and wherein the numerical value of R is in the interval from and including 0 to less than 0.6.

In another aspect the invention pertains to a material having a first contact angle and comprising a substratum having a second contact angle, said substratum being contacted by a macromolecule, wherein the relation between said first and second contact angle as defined by the ratio between

i) the difference between said second contact angle, when no macromolecule is present, and said first contact angle, and

ii) the difference between said second contact angle, when no macromolecule is present, and the contact angle of said substratum, when said substratum is saturated by said macromolecules as defined herein,

is more than -0.6 and less than 0.6.

In another aspect the invention pertains to a material having a first contact angle and comprising a substratum having a second contact angle, said substratum being contacted by a plurality of soluble substances capable of forming a self-assembled monolayer comprising a macromolecule and having a third contact angle, wherein the relation between said contact angles as defined by the ratio between

i) the difference between the third contact angle of said monolayer, when no macromolecule is present, and said first contact angle, and

ii) the difference between the third contact angle of said monolayer, when no macromolecule is present, and the contact angle of said self-assembled monolayer, when said monolayer is saturated by said macromolecules as defined herein,

is more than -0.6 and less than 0.6.

All contact angles used to characterize the material are advancing contact angles. Pure water is used as probing liquid, and air saturated with water vapor, is used as probing gas. The material/substratum, the pure and furthermore double distilled water and the air saturated with water vapor, will form the three-phase boundary used to measure the contact angle.

The described properties of a biocompatible surface according to the invention comprising said hydrophobic substratum and said hydrophilic macromolecule allows a first determinant to adhere to and remain associated with said surface in a functional conformation or a biologically active form or conformation. The properties of said surface comprising said substratum and said macromolecule and said first determinant are also useful, as a second determinant can adhere to and remain associated in a functional or active form or conformation, preferably a biologically active form or conformation, with said first determinant and consequently with the surface.

### Figure Captions

Figure 1 An adsorbed/immobilized biologically active moiety (e.g. a polypeptide) becomes conformationally altered (with time) and thus inactive due to attractive (e.g. hydrophobic) interactions between the underlying substratum and the adsorbed/immobilized polypeptide

Figure 2 A schematic showing how to measure contact angles at a three phase boundary, i.e. substratum (solid), water (liquid), and water vapour (gas). A bubble of water vapour is captivated by the above horizontal substratum which is immersed into water.

Figure 3 Immobilized hydrophilic macromolecules neighboring interstitially adsorbed/immobilized moities (e.g. a polypeptide) exert a lateral pressure upon said moities stabilizing them in their active conformation

Figure 4 A two-step polymer surface functionalization procedure

- Figure 5 Hydrophilic macromolecules, neighboring interstitially adsorbed/immobilized moities, are immobilized to the underlying substratum by means of chemical bonds.
- 5 Figure 6 Hydrophilic macromolecules, neighboring interstitially adsorbed/immobilized moities, are immobilized to the underlying substratum by means of ionic bonds (countercharges).
- Figure 7 Hydrophilic macromolecules, neighboring interstitially adsorbed/immobilized moities, are immobilized to the underlying substratum by means of adsorption.
- 10 Figure 8 Hydrophilic macromolecules, neighboring interstitially adsorbed/immobilized moities, are immobilized to the underlying polymer substratum by means of mutual entanglement.
- Figure 9 Hydrophilic macromolecules, neighboring interstitially adsorbed/immobilized moities, are immobilized to an underlying SAM.
- 15 Figure 10 Adsorption kinetics of ABMPEG 5 kDa and MPEG 5 kDa out of aqueous solutions (10 g/l) onto PSf, spin-coated on polished silica wafers, as determined by ellipsometry.
- Figure 11 Advancing and receding CA on PSf, spin-coated on glass cover slips and being modified at different ABMPEG 10 kDa bulk concentrations.
- 20 Figure 12 Receding CA and their hysteresis on PSf, spin-coated on glass cover slips and being modified at different concentrations of ABMPEG 10 kDa, ABMPEG 5 kDa, and ABMPEG 2 kDa.
- Figure 13 Table of CA-hysteresis and receding CA relating to Figure 12 (n.d.: no data available).
- 25 Figure 14 Advancing and receding CA on PSf, spin-coated on glass cover slips and being modified with solution mixtures of ABMPEG 2 kDa and ABMPEG 10 kDa yielding a total ABMPEG concentration of 10 g/l.

- Figure 15 Receding CA on PSf, spin-coated on glass cover slips and being modified at different concentrations of ABMPEG 10 kDa. CA are shown after modification and after consecutive rinse with isopropanol/water = 1/1.
- 5 Figure 16 Adsorbed amount of BSA on an unmodified and ABMPEG 5 kDa modified PSf UF membrane after 2 h static exposure of the membrane to a 1 g/l BSA solution (0.15 molar phosphate buffer, pH = 7, room temperature) and consecutive gentle rinsing in buffer.
- 10 Figure 17 Adsorption kinetics of FN to unmodified and ABMPEG 10 kDa modified PSf, spin-coated on polished silicon wafers, as monitored by in-situ ellipsometry
- 15 Figure 18 Overall cell morphology of HF adhering on unmodified PSf or on ABMPEG 10 kDa modified PSf, spin-coated on glass cover slips. Effect of ABMPEG 10 kDa density. HF were plated for 2 h on unmodified PSf (A), or PSf grafted at different ABMPEG 10 kDa concentrations as follows: (B) 0.001 g/l, (C) 0.01 g/l, (D) 0.1 g/l, (E) 1 g/l, (F) 10 g/l. At the end of incubation, samples were investigated and photographed under phase contrast at low magnification (20X).
- 20 Figure 19 Number of adherent HF per microscopic field on unmodified PSf and ABMPEG 10 kDa modified PSf, spin-coated on glass cover slips. Error bars represent standard deviations of the obtained data.
- 25 Figure 20 Focal adhesion formation of HF adhering on unmodified PSf and ABMPEG 10 kDa modified PSf, spin-coated on glass cover slips. Effect of ABMPEG 10 kDa density. HF were plated for 2 h on unmodified PSf (A), or PSf grafted at different ABMPEG 10 kDa concentrations as follows: (B) 0.001 g/l, (C) 0.01 g/l, (D) 0.1 g/l, (E) 1 g/l, (F) 10 g/l. At the end of incubation, the cells were fixed, permeabilized and stained for vinculin by immunofluorescence. Samples were visualized and photographed at high magnification (100X).

- Figure 21 Focal adhesion formation of HF adhering on unmodified PSf and ABMPEG 10 kDa modified PSf, spin-coated on glass cover slips. Effect of serum pre-coating. HF were plated for 2 h on serum-coated unmodified PSf (A), or on serum-coated PSf grafted at different ABMPEG 10 kDa concentrations as follows: (B) 0.001 g/l, (C) 0.01 g/l, (D) 0.1 g/l, (E) 1 g/l, (F) 10 g/l. At the end of incubation, the samples were fixed, permeabilized and stained for vinculin. Samples were visualized and photographed at high magnification (100X).
- Figure 22 FN matrix formation by HF cultured on unmodified PSf and ABMPEG 10 kDa modified PSf, spin-coated on glass cover slips. Effect of PEG density. HF were cultured for 5 days in DMEM containing 10% FBS on: (A) unmodified PSf, or on modified PSf grafted at different ABMPEG 10 kDa concentrations as follows, (B) 0.001 g/l, (C) 0.01 g/l, (D) 0.1 g/l, (E) 1 g/l and (F) 10 g/l. At the end of incubation, the HF were fixed and stained for FN by immunofluorescence. Samples were viewed and photographed at low magnification (25X).
- Figure 23 FN matrix formation by HF cultured on unmodified PSf and on ABMPEG 10 kDa modified PSf surfaces. Effect of ABMPEG 10 kDa density. HF were cultured for 5 days in DMEM containing 10% FBS on: (A) unmodified PSf, or on PSf grafted at different ABMPEG 10 kDa concentrations as follows: (B) 0.001 g/l, (C) 0.01 g/l, and (D) 10 g/l. At the end of incubation, the HF were fixed and stained for FN by immunofluorescence. Samples were viewed and photographed at high magnification (100X).
- Figure 24 HF proliferation on unmodified PSf and ABMPEG 10 kDa modified PSf, spin-coated on glass slides. Phase contrast photographs were taken at 1, 3, and 7 days.
- Figure 25 HUVEC proliferation on unmodified PSf and ABMPEG 10 kDa modified PSf, spin-coated on glass slides. Phase contrast photographs were taken at 3, 5, and 7 days.

- Figure 26 C3A proliferation on unmodified PSf and ABMPEG 10 kDa modified PSf, spin-coats on glass slides. Phase contrast photographs were taken at 3, 5, and 7 days.
- 5 Figure 27 XTT assay for HF after 1, 3, and 7 days, cultivated on unmodified PSf and on ABMPEG 10 kDa modified PSf, spin-coated on glass slides and compartmented into 8 wells by silicon masks. Error bars represent the standard deviation of the data.
- 10 Figure 28 LDH assay for HF after 1, 3, and 7 days, cultivated on unmodified PSf and on different ABMPEG 10 kDa modified PSf, spin-coated on glass slides and compartmented into 8 wells by silicon masks. Error bars represent the standard deviation of the data.
- 15 Figure 29 Focal adhesion formation of HUVEC adhering on unmodified and ABMPEG 10 kDa modified PSf, spin-coated on glass cover slips. HUVEC were plated for 2 h on FN-coated unmodified PSf (A), or on FN-coated PSf grafted at different ABMPEG 10 kDa concentrations as follows: (B) 0.001 g/l, (C) 0.01 g/l, (D) 0.1 g/l, (E) 1 g/l, (F) 10 g/l. At the end of incubation, the samples were fixed, permeabilized and stained for vinculin. Samples were visualized and photographed at high magnification (100X).
- 20 Figure 30 Ellipsometric data of the consecutive adsorption of i) BGG as antigen, ii) HSA as blocking agent, and iii) a-BGG as respective antibody to BGG to unmodified PSf and PSf modified with ABMPEG 10 kDa at a concentration of 10 g/l. PSf was previously spin-coated on polished silicon slides. Arrows indicate flushing with buffer or addition of concentrates as described in the text.
- 25 Figure 31 Figure a-c shows ellipsometric data of the consecutive adsorption of a) BGG as antigen, b) HSA as blocking agent, and c) a-BGG as respective antibody to BGG, to unmodified PSf and PSf modified with ABMPEG 10 kDa at different concentrations. All values are arithmetic means of two independent experimental runs. Error bars represent the
- 30

respective standard deviations. PSf was previously spin-coated on polished silicon slides. Figure 31d shows the ratio between the adsorbed amount of a-BGG in the third step (c) and the bound amount of BGG in the first step (a).

5

### **Detailed description of the invention**

The following definitions are being used to illustrate the present invention.

#### **Definitions**

10 **Active conformation:** protein in a conformation, where it has its normal biological activity in a native host organism.

**Active form:** protein or biological material in a form, where it has the same function as when said protein or biological material is present in native host or native environment.

15 **Adsorption:** the taking up of molecules from a gas or liquid on the surface of another substance such as a substratum.

**Advancing contact angle:** contact angle when the liquid front is caused to advance over said solid material/substratum.

**Amphiphil:** substance containing both polar, water-soluble and nonpolar, water-insoluble groups.

20 **Arrays for culture of "sensual" cells:** Solid or semi-solid supports with ordered structures for the attachment of sensual cells, such as retina cells.

**Biocompatible material:** Material that, when interacting with biological material, does not induce an acute or chronic inflammatory response and does not prevent a proper differentiation of implant-surrounding tissues.

25 **Biologically active form:** see active form.

**Biologically active conformation:** see active conformation.



**Biological material:** Any material derived from a living entity including plants, animals or a living part thereof, such as an organ or cell. The preferred biological system is a mammalian system, preferably a human system.

5 **Biomaterial:** A material interfacing with biological systems to e.g. evaluate, treat, augment or replace any tissue, organ or function of the body.

**Biogenic ligand:** Any ligand of biological origin, such as carbohydrates, proteins or parts thereof such as e.g. oligopeptides, including any combination and/or derivatives thereof.

10 **Biohybrid organ:** A device comprising a combination of a biomaterial and a biological material in an active form, such as e.g. specific organ cells.

**Cell differentiation:** Process by which a precursor cell becomes a distinct specialized cell type.

**Conformational alterations:** Change in the overall three dimensional form of a material, usually a biological material.

15 **Conformational entropy:** The entropy of a macromolecule as determined by the amount of possible conformations that the macromolecule may attain.

**Conjugate:** Plurality of functional molecules chemically joined together.

20 **Contact angle (CA):** Angle ( $\theta$ ) represented by the limits of the liquid phase at a three phase boundary between a solid or semi-solid surface, a liquid and the saturated vapour of said liquid. Different methods are applied to generate a three phase boundary, as e.g. the captive bubble method, where a bubble of saturated vapour of the used test liquid is captivated by the test surface. With respect to the claims in this invention, it is the advancing contact angle which characterizes the material surfaces.

25 **Deactivation:** Alteration of an active form or conformation to a less active form or conformation.

**Density:** Mass per volume (concentration) or per area (lateral density).

**End group:** Distal part of a macromolecule.

**Excluded volume:** Interaction between segments of solvated macromolecules or polymer chain(s) that are moving to occupy the same space.

5 **Extracellular matrix (ECM):** Meshwork synthesized by cells and composed of adhesive proteins such as glycoproteins, laminin, FN, interconnected collagen fibrils, hyaluronate and proteoglycans as structural and functional support of tissue cells.

**Film:** Synthetic material in the form of long, thin sheets.

**Flexible:** Capable of attaining many conformations, in contrast to rigid.

**Flux:** Measure of the flow of some quantity per unit area per unit time

10 **Functionalization:** Chemical derivatization changing structure, properties and/or function.

**Grafting:** Attaching at least one macromolecule comprising equal or different molecular units to a substratum through a chemical bond.

15 **Head group:** Proximal group, the group forming the link between a macromolecule and a substratum.

**Hydrophilic polymer:** Any polymer with a high surface energy where droplets of water spread readily.

**Hydrophobic polymer:** Any polymer with low surface energy where water forms prominent droplets on the surface.

20 **Improved contact:** Enhanced attachment and spreading of cells upon contact with non-biological supports.

**Interface:** Area or surface that represents the boundary between two separate phases of a chemical or physical process.

25 **Ionic bond:** Bond held together by coulombic interactions between differently charged moieties.

**Latent:** Present but not (yet) active.

**Laterally structured monolayer:** Monolayer formed of macromolecules interacting with neighboring molecules due to their inherent excluded volume, to spontaneously form a relatively ordered array of macromolecules, said monolayer is not crystalline and characterized by a water content of at least 50 percent.

**Layer density:** Mass per area (2D concentration).

**Linker:** Connects two moieties or groups or molecules with each other.

**Macromolecule:** Any molecule having a MW higher than 400 Da.

**Membrane:** Barrier between two phases and allowing transport via sorption/diffusion and/or through pores.

**Permeability:** Measure of the capability of a membrane to allow transport through said membrane.

**Photo:** Physical stimulus, here to initiate a chemical reaction.

**Photo-reactive polymer:** Polymer comprising one or more latently reactive groups.

**Polymer:** Molecule formed by the union of at least five identical monomers

**Pretreatment:** The addition of functional groups to a substratum.

**Receding contact angle:** Contact angle when the liquid front is caused to recede over said solid.

**Refractive index:** Ratio of the phase velocity of electromagnetic radiation in a vacuum (or air) to that in a transparent medium.

**Rigid:** Essentially non-flexible.

**Saturated substratum:** Saturation of a substratum is attained, when the contact angle of said substratum contacted by a plurality of macromolecules can not be further reduced by adding further macromolecules to the surface of the substratum. More preferably, saturation of a substratum is attained, when no significant change of the

contact angle can be achieved when said substratum is being contacted by a plurality of macromolecules.

**Self-assembled monolayer:** Monolayer formed on a substratum and comprising self-assembled (stacked or crystallized) components comprising a headgroup, said headgroup interacting favorably with the substratum, and an endgroup, said endgroup being orientated towards the solution. Said monolayer is characterized by a crystalline, highly ordered structure and a very low water content or substantially no water content.

**Solvated:** Molecule or material being in solution.

**Synthetic material:** Any material that is not of biological origin.

**Substratum:** Any chemical moiety to which macromolecules are capable of attaching.

**Surface:** Outer part of an object, here the biomaterial or its precursor.

15

The present invention teaches a new way of controlling cell adhesion and biocompatibility of polymer substratum surfaces associated therewith. The novel approach is based on a structuring of a hydrophobic substratum surface, preferably a hydrophobic polymer substratum, with a layer of macromolecules, preferably a monomolecular layer of flexible macromolecules, more preferably a monolayer of laterally patterned macromolecules contacted with said surface of said hydrophobic polymer substratum.

20

25

The response of essentially biological systems to the designed surfaces according to the invention is different from the response of such systems to the polymers of the prior art. Protein adsorption, antibody binding to adsorbed antigens as well as studies with fibroblasts, endothelial cells, keratinocytes, liver cells and others— i.e. biological materials well accepted as a general cellular model for tissue-biomaterial interaction - have been carried out in order to evaluate the ability of the novel biocompatible

surfaces to support and/or improve the function of biological material brought in contact with it. The ability of cells to attach, spread and proliferate on various surfaces that had been modified according to the invention is of particular importance in that context. The production of an extracellular matrix is one of several key functions of fibroblasts and generally a characteristic feature of cells of the connective tissue type. Consequently, the ability of cells to attach to biomaterials according to the invention has been studied by microscopical investigations of extracellular matrix formation i) within the first hours of cell attachment, by means of fluorescently labeled FN, and ii) following long-term culture through direct detection of the synthesized FN matrix.

The studies revealed an improved cellular functionality as a function of e.g. the MW of the macromolecules attached to the polymer substratum, and the degree of surface functionalization. The results were measured by typical biocompatibility parameters such as e.g. cell adhesion and morphology, formation of focal adhesions points, the formation of an extracellular matrix, and the effect onto the long-term proliferation. All of the above is understood to contribute to the observed improved cellular functionality as defined herein above. In other words, the results clearly showed that polymer substratum surfaces modified according to the invention has a superior functionality. The functionality is superior when compared to both the original, unmodified polymer, and to the fully modified or "coated" surfaces of the prior art that are characterized by a comparatively high degree of surface functionalization.

The results described herein are strong indications that it is possible to further optimize the relationship that exists between adsorption of essentially biological material, the state, or conformation, or biologically active form of said adsorbed material, and the cellular behavior or functionality resulting from said adsorption.

Consequently, the invention makes it possible to determine empirically one or more optima of cellular functionality by means of a rational design approach that is readily controllable by any suitable state of the art physico-chemical surface analysis. Hence, the present invention achieves its objective by significantly improving state of the art methods of providing biomaterials, since the response of adsorbed cells and their biocompatibility can now be predetermined or at least designed quickly and

economically by well-defined and readily adjustable state of the art physico-chemical and bioengineering parameters.

Materials that are capable of being processed according to the invention are those with  
5 at least suitable, if not superior physico-chemical properties for any given application, such as e.g. suitable or superior properties like transparency, refraction index, electrical conductivity, thermal stability, hydrolytic resistance, or membrane forming properties (ranging from, e.g. cell-culture dishes to membranes), but are currently less than adequate, if not entirely useless, for the attachment, growth and function of cells  
10 because of their undesirable physico-chemical surface properties.

The surface structures of the biomaterials to be processed in accordance with the invention may be porous structures with a stochastic or predetermined or controlled permeability (e.g. micro- or macro-porous flat-sheet or hollow-fiber membranes) that  
15 may be built up as a temporary or permanent support of cells described herein immediately below.

The two-step modification technique disclosed herein (see Fig.4) preferably generates a covalently bound, patterned molecular monolayer. The structure or functionality of  
20 the layer may be designed or predetermined by synthesis of macromolecule conjugates and then in a first adsorptive step according to any given set of particular circumstances. By covalent grafting, a stable attachment (i.e. grafting) to the underlying polymeric material (basis polymer) is readily achieved. The control of the "design parameters" such as e.g. molecular structure of the amphiphilic  
25 macromolecule, the concentration and/or solvency of said macromolecule can be left to a person skilled in the art of manufacturing complex polymers.

MW and/or size of the amphiphil determines at least to some extent the molar density (i.e. macromolecules per surface area). An increased interaction between the  
30 amphiphilic macromolecule and the polymer substratum is likely to lead to an increased layer density. Likewise, a high concentration of amphiphilic macromolecules in the first step (see Fig.4), or a decreased solvency of said amphiphilic macromolecules will also contribute to an increased layer density.

Changes in solvency may be attainable through variations in e.g. salt concentration, pH, temperature or polarity of the solvent. Application of the amphiphiles by spray-coating and subsequent drying followed by ultra violet (UV) or visible (Vis) irradiation can be alternative technologies. The person skilled in the art is familiar with the physical chemistry of polymers and macromolecules required in order to attain an altered layer density.

It is understood that when the biomaterial is a film, the polymer substratum is substantially impenetratable to water, whereas the polymer substratum is porous, when the biocompatible material is a membrane.

The created lateral layer structure according to the invention is characterized by the amphiphil nature of the macromolecule and amphiphil-amphiphil intermolecular and intramolecular interactions. Strong repulsive interactions between the amphiphiles due to their inherent large excluded volumes lead to discretely adsorbed molecules capable of forming a laterally "self-assembled" structure.

According to one preferred embodiment of the invention, surface functionalization is mediated by well-defined photo-reactive conjugates of hydrophilic, flexible macromolecules comprising a modular composition of building blocks. In one particularly preferred embodiment said modular composition comprises:

*(Latent-reactive head-group)-(guiding-group)-(main body)-(functional end-group)*

The invention aims to provide a substratum surface with desired physical characteristics and comprises the steps of contacting the substratum with a composition comprising a plurality of macromolecules possessing desired physical characteristics. The macromolecules each comprise covalently bonded, optionally via a linker group, to their main body, a latent-reactive head-group, and optionally also a guiding group, and a functional end-group. The latent-reactive head-group is capable of providing one or more active species such as free radicals in response to external stimulation to covalently bind the macromolecules to the substratum, through the residues of the latent-reactive head-group.

The macromolecule is spatially oriented so as to enable one or more of its latent-reactive groups to come into covalent bonding proximity with the substratum surface, and the method according to the present invention includes the further step of activating the latent-reactive groups by applying external stimulation to covalently bond the macromolecule to the substratum. The external stimulation that is employed is preferably electromagnetic radiation, and more preferably the radiation is in the ultraviolet, visible or infra-red regions of the electromagnetic spectrum, since the layer structure established by "self-assembly" is not disturbed by this kind of radiation, and the polymer substratum is left at least substantially intact. The degree of conversion is selectable by e.g. UV/Vis dose, and typically 100% conversion will be attempted. The response to the activation step of the method can be tuned by selecting different latent-reactive groups. Also, the reactivity of the photo-chemically generated reactive species can be selected in accordance to the structure of the polymer substratum. Thus, it is well known that e.g. aryl nitrenes from aryl azides will react via insertion reactions with all polymers having -NH, -OH or -CH groups, and aromatic ketones after UV/Vis excitation will undergo a hydrogen abstraction eventually leading to an insertion reaction with all polymers having at least -CH groups

The latent-reactive head-group of a macromolecule employed in the invention may comprise one or more covalently bonded latent-reactive groups. The latent-reactive groups, as defined herein, are groups which respond to specific applied external stimuli to undergo an active species generation resulting in covalent bonding to an adjacent support surface. Latent-reactive groups are those groups of atoms in a molecule which retain their covalent bonds unchanged under conditions of storage but which, upon activation, form covalent bonds with other molecules. The latent-reactive groups generate active species such as free radicals, nitrenes, carbenes, and excited states of ketones upon absorption of external electromagnetic or kinetic (thermal) energy. Latent-reactive groups may be chosen to be responsive to various portions of the electromagnetic spectrum, and latent-reactive groups that are responsive to ultraviolet, visible or infrared portions of the spectrum are preferred.



The azides constitute a preferred class of latent-reactive groups and include arylazides such as phenyl azide, 4-azido benzoic acid, and 4-fluoro-3-nitrophenyl azide, acyl azides such as benzoyl azide and p-methylbenzoyl azide, azido formates such as ethyl azidoformate, phenyl azidoformate, sulfonyl azides such as benzenesulfonyl azide, and phosphoryl azides such as diphenyl phosphoryl azide and diethyl phosphoryl azide. Diazo compounds constitute another class of latent reactive groups and include diazoalkanes ( $-\text{CHN}_2$ ) such as diazomethane and diphenyldiazomethane diazoketones such as diazoacetophenone and 1-trifluoromethyl-1-diazo-2-pentanone, diazoacetates such as t-butyl diazoacetate and phenyl diazoacetate, and beta-keto-alpha-diazoacetates such as t-butyl alpha diazoacetoacetate. Other latent-reactive groups include the aliphatic azo compounds such as azobisisobutyronitrile, the diazirines such as 3-trifluoromethyl-3-phenyldiazirine, the ketenes ( $-\text{CH}=\text{C}=\text{O}$ ) such as ketene and diphenylketene and photoactivatable ketones such as benzophenone and acetophenone. Peroxy compounds are contemplated as another class of latent-reactive groups and include dialkyl peroxides such as di-t-butyl peroxide and dicyclohexyl peroxide and diacyl peroxides such as dibenzoyl peroxide and diacetyl peroxide and peroxyesters such as ethyl peroxybenzoate.

Upon activation of the latent-reactive groups to cause covalent bond formation to the surfaces to which macromolecules are to be attached, the macromolecules are covalently attached to the surfaces by means of residues of the latent reactive groups.

As will be noted from the foregoing disclosure, photoreactive groups are for the most part aromatic and are hence generally hydrophobic rather than hydrophilic in nature. The presence of a comparatively hydrophobic reactive head-group such as an aromatic photoreactive group, appears to be causing the macromolecule to orient itself in an aqueous solution with respect to a hydrophobic substratum surface such that the comparatively hydrophobic reactive head-group is preferentially carried near the support surface while the remainder of the macromolecule, i.e. the main body and the functional end-group, is generally orientated away from the hydrophobic substratum surface. It is known that this feature enables macromolecules to be covalently bonded

densely to a comparatively hydrophobic support substratum surface, and this in turn contributes to the formation of a biocompatible substratum surface as defined above.

5 According to the above, the amphiphilic character and thus orientation and achieved  
grafting density of macromolecules to a substratum surface can be increased by  
incorporating a hydrophobic guiding-group into the macromolecule. The guiding-  
group is a bifunctional group that is positioned, preferably by means of a linker group,  
between the latent-reactive head-group and the remainder of the macromolecule, i.e.  
the main body and the functional end-group. The guiding-group is hydrophobic for the  
10 purpose of enhancing the preferential orientation of the latent-reactive head-group of  
the macromolecule into bonding proximity of the substratum surface and for the  
purpose of increasing the amphiphilic character of the macromolecule in order to  
increase the achieved grafting density. Preferred classes of guiding groups are  
aliphatic, linear or weakly branched groups or cyclic aliphatic groups, both preferably  
15 with from 6 to 18 carbon atoms, or combinations thereof, as well as mono- or  
polycyclic aromatic groups, or their combinations with the above-mentioned aliphatic  
groups.

The main body of the macromolecule is preferably hydrophilic, uncoiling in an  
20 aqueous environment and thus exhibiting an excluded volume. It may be a polymer of  
natural or synthetic origin. Such polymers include oligomers, homopolymers and  
copolymers resulting from addition or condensation polymerization, and natural  
polymers including oligosaccharides, polysaccharides, oligosaccharides, and  
polypeptides or a part thereof, such as an extended oligopeptide. The polymer forming  
25 the main body may comprise several distinct polymer types, as prepared by terminal  
or side chain grafting, including cellulose-based products such as hydroxyethyl  
cellulose, hydroxypropyl cellulose, carboxymethyl cellulose, cellulose acetate and  
cellulose butyrate, acrylics such as those polymerized from hydroxyethyl acrylate,  
hydroxyethyl methacrylate, glyceryl acrylate, glyceryl methacrylate, acrylic acid,  
30 methacrylic acid, acrylamide and methacrylamide, vinyls such as polyvinyl  
pyrrolidone and polyvinyl alcohol, nylons such as polycaprolactam, polylauryl lactam,  
polyhexamethylene adipamide and polyhexamethylene dodecanediamide;  
polyurethanes, polylactic acids, linear polysaccharides such as amylose, dextran,

chitosan, and hyaluronic acid, and branched polysaccharides such as amylopectin, hyaluronic acid and hemi-celluloses.

5 The macromolecules themselves preferably have MWs of at least about 500 Da, most preferably of about 10.000 Da, and are hydrophilic in nature, and soluble in water to the extent of at least approximately 0.5 % by weight at 25°C.

10 In a preferred embodiment the main body comprises repeating units as e.g. ethoxy (-CH<sub>2</sub>-CH<sub>2</sub>-O-) or isopropoxy (-CH<sub>2</sub>-CH(CH<sub>3</sub>)-O-) groups, and of these PEG is most preferred.

15 Functional endgroups include all chemical moieties that can be used to link permanently or reversibly other biological or synthetic molecules or cells, viruses and the like via the polymeric main body to a surface, such as hydroxy, amino, carboxyl, sulphonic acid, activated esters, or epoxy groups as well as charged or chelating functionalities.

20 Additionally, the functional end-group may be chosen from a wide variety of compounds or fragments thereof which will render the modified substratum generally or specifically "biophilic" as those terms are defined below. Generally biophilic functional end-groups are those that would generally promote the binding, adherence, or adsorption of biological materials such as, for example, intact cells, fractionated cells, cellular organelles, proteins, lipids, polysaccharides, simple carbohydrates, complex carbohydrates, and/or nucleic acids. Generally biophilic functional end-  
25 groups include hydrophobic groups or alkyl groups with charged moieties such as -COO<sup>-</sup>, -PO<sub>3</sub>H<sup>-</sup> or 2-imidazolo groups, and compounds or fragments of compounds such as extracellular matrix proteins, FN, collagen, laminin, serum albumin, polygalactose, sialic acid, and various lectin binding sugars. Specifically biophilic functional end-groups are those that selectively or preferentially bind, adhere or  
30 adsorb a specific type or types of biological material so as, for example, to identify or isolate the specific material from a mixture of materials. Specific biophilic materials include antibodies or fragments of antibodies and their antigens, cell surface receptors and their ligands, nucleic acid sequences and many others that are known to those of

ordinary skill in the art. The choice of an appropriate biophilic functional end-group depends on considerations of the biological material sought to be bound, the affinity of the binding required, availability, facility of ease, and cost. Such a choice is within the knowledge, ability and discretion of one of ordinary skill in the art.

5

For the preparation of biodegradable coatings or coatings that may be degraded under predefined environmental conditions, it is desirable to incorporate in the macromolecule a moiety that allows either enzymatic or chemical hydrolysis of the coating. Suitable ingredients include amino acids such as alanine, valine, leucine, 10 proline, methionine, aspartic acid, threonine, serine, glutamic acid, glycine, cysteine, phenylalanine, lysine, histidine, argine, and aminobutyric acid. Alternatively, hydrolytically unstable ester bonds can be applied as well. All these moieties are typically part of a linker group, when such a group is present, but may also be incorporated into the main-body or the guiding-group of the macromolecule.

15

The lateral density of the monolayer of macromolecules according to the invention is adjustable by e.g. i) modification of the amount and/or concentration of macromolecules in solution during "self-assembly", or ii) the use of mixtures of macromolecules, said macromolecules comprising varying building blocks as e.g. 20 different MWs (MW), or variations in other structural features of the macromolecule (e.g. branched vs. unbranched), or iii) adjustable by appropriately choosing solution conditions during an adsorptive application of said macromolecules, as e.g. the solvency, the ionic strength, the temperature or the pH. The process of photochemical grafting does neither disturb this "self-assembled" pattern, nor does it result in any 25 substantial degradation of the underlying surface of the polymer substratum.

The substratum comprises a definable surface such as the tangible surface of film or a membrane, or the surface of a contact lens or surgical implant, or the surface provided by small particles in an emulsion or other suspension or as a powder, or as the surface 30 of a soft gel. The invention provides the particular advantage of providing means by which non-pretreated definable (e.g., solid) surfaces may simply and rapidly be provided with covalently bonded macromolecular coatings in a simple, rapid and hence economical manner.

Preferred embodiments of the invention are described herein below. The material according to the invention may comprise soluble substance in the form of molecules capable of forming a self-assembled monolayer. Also, the substratum may be pretreated or modified, preferably as the result of said substratum being contacted by and/or operably linked to a charged group or a hydrophilic compound.

As defined above, the contact angle of said material is an advancing contact angle. In one embodiment, the advancing contact angle is in the range of from 50 degrees to 140 degrees, preferably in the range of from 60 degrees to 125 degrees, such as in the range of from 70 degrees to 120 degrees, for example in the range of from 75 degrees to 110 degrees, such as in the range of from 80 degrees to 100 degrees.

However, a material also exhibits a receding contact angle, in which case the contact angle is in the range of from 30 degrees to 120 degrees, preferably in the range of from 40 degrees to 110 degrees, such as in the range of from 50 degrees to 100 degrees, for example in the range of from 60 degrees to 90 degrees, such as in the range of from 70 degrees to 80 degrees.

The ratio between the difference between said second contact angle, when no macromolecule is present, and said first contact angle, and the difference between said second contact angle, when no macromolecule is present, and the contact angle of said substratum, when said substratum is saturated by said macromolecules as defined herein, is more than -0.6 and less than 0.6, and preferably in the range of from 0 to less than 0.50, such as less than 0.40, for example less than 0.30, such as less than 0.25, for example less than 0.20, such as less than 0.15, for example less than 0.10, such as less than 0.05.

When the contact angle is the receding contact angle the ratio is preferably less than 0.40.

The ratio between the difference between the third contact angle of said monolayer, when no macromolecule is present, and said first contact angle, and the difference between the third contact angle of said monolayer, when no macromolecule is present, and the contact angle of said self-assembled monolayer, when said monolayer is saturated by said macromolecules as defined herein, is more than -0.6 and less than 0.6, and preferably in the range of from 0 to less than 0.50, such as less than 0.40, for example less than 0.30, such as less than 0.25, for example less than 0.20, such as less than 0.15, for example less than 0.10, such as less than 0.05.

In one particularly preferred embodiment there is provided a material which, when contacted by a first determinant comprising a compound selected from the group consisting of a polypeptide, or part thereof, a nucleic acid moiety, a carbohydrate moiety, and a lipid moiety, including any combination thereof, is capable of maintaining said compound in a biologically active form. More preferably the compound is a polypeptide or part thereof.

There is also provided a material further comprising said first determinant comprising said compound, wherein said first determinant is maintained in a biologically active form when contacted by said substratum and/or said macromolecule. The biologically active form is preferably an essentially biologically active conformation. The biologically active form or conformation is preferably maintained and/or improved and/or stabilized by means of the cooperativity of said substratum and said macromolecule. The biologically active form or confirmation is preferably maintained and/or improved and/or stabilized when contacted by said substratum and said macromolecule. The material according to the invention is preferably biocompatible.

There is also provided a material according to the invention, wherein the weight increase per area unit arising from the part of the macromolecule essentially consisting of PEG or poly(ethylene oxide) (PEO) is less than  $2.0 \times 10^{-22}$  grams (g) per square nanometer ( $\text{nm}^2$ ), such as less than  $1.0 \times 10^{-22}$  grams (g) per square nanometer ( $\text{nm}^2$ ), for example less than  $0.8 \times 10^{-22}$  grams (g) per square nanometer ( $\text{nm}^2$ ), such as less than  $0.5 \times 10^{-22}$  grams (g) per square nanometer ( $\text{nm}^2$ ), for example less than  $0.3 \times 10^{-22}$  grams (g) per square nanometer ( $\text{nm}^2$ ).

There is also provided a material wherein the substratum is contacted by a plurality of soluble compounds capable of forming a layer of self-assembled macromolecules, preferably n-alkane chains preferably containing from 8 to 24 carbons. The macromolecule according to the invention can be characterized by an excluded volume.

The substratum preferably comprises a hydrophobic polymer and in one embodiment the substratum is at least substantially flexible and/or a film. However, the substratum may also be essentially rigid or at least substantially non-flexible. In this case, the substratum may comprise a crystalline structure capable of supporting a self-assembled monolayer such as gold, silicon oxide, and similar crystalline structures and/or structures that are smooth on a nanometer scale.

The macromolecule according to the invention comprises a hydrophilic polymer or an amphiphilic polymer. The macromolecule preferably has a MW of more than 400 Da, such as a MW of more than 1,000 Da, for example a MW of more than 5,000 kDa, such as a MW of more than 10,000 Da, for example a MW of more than 50,000 Da, such as a MW of more than 100,000 Da.

The macromolecule according to the invention is preferably a conjugate comprising a head group, a guiding group, a linker group, a polymer chain or a main body, and a functional end group.

The head group is capable of forming a chemical bond (see Fig.5), such as a ionic bond (see Fig.6), and may adsorb to the substratum (see Fig.7) or be entangled into or with the substratum (see Fig.8). The head group may also be capable of forming a self-assembled monolayer (see Fig.9).

A preferred guiding group is a bifunctional group comprising an aliphatic, linear or weakly branched group. The guiding group may also be capable of forming and/or stabilizing a self-assembled monolayer.

A preferred linker group is capable of being enzymatically or chemically hydrolyzed, it may be hydrolytically unstable, or it may be essentially stable against cleavage under practical circumstances.

- 5     The polymer chain or main body is preferably hydrophilic, uncoiling in an aqueous environment and exhibiting an excluded volume.

The functional end group is capable of linking permanently or reversibly other biological or synthetic molecules or materials.

10

- A first determinant as defined herein comprises a biologically active compound comprising a polypeptide, or a part thereof, a nucleic acid moiety, a carbohydrate moiety, and a lipid moiety, including any combination thereof. The biologically active compound is preferably selected from the group consisting of membrane associated and/or extracellular matrix polypeptides natively produced by a microbial cell, a plant cell or a mammalian cell. The biologically active compound in another embodiment is selected from the group consisting of a polypeptide, an antibody, a polyclonal antibody, a monoclonal antibody, an immunogenic determinant, an antigenic determinant, a receptor, a receptor binding protein, an interleukine, a cytokine, a cellular differentiation factor, a cellular growth factor, and an antagonist to a receptor.
- 15
- 20

- The biologically active compound may also be a synthetic polypeptide, or part thereof, capable of contacting said substratum and/or said macromolecule. Preferably the biologically active compound is an adhesion polypeptide, preferably FN or vitronectin.
- 25

- The biologically active compound preferably results in an improved contact between said material and a biological entity, such as a biological cell or a virus, or part thereof, including a polypeptide, or a part thereof, a nucleic acid moiety, a carbohydrate moiety, and a lipid moiety, including any combination thereof.
- 30

In one particularly preferred embodiment the material according to the invention further comprises a second determinant as defined herein. The second determinant



comprises a biological entity, such as a biological cell or a virus, or part thereof, including a polypeptide, or a part thereof, a nucleic acid moiety, a carbohydrate moiety, and a lipid moiety, including any combination thereof.

5 The biological entity is preferably also selected from the group consisting of a polypeptide, an antibody, a polyclonal antibody, a monoclonal antibody, an immunogenic determinant, an antigenic determinant, a receptor, a receptor binding protein, an interleukine, a cytokine, a differentiation factor, a growth factor, and an antagonist to the receptor. The biological cell, or part thereof, is preferably a  
10 mammalian cell, including a human cell and an animal cell, a plant cell, a microbial cell, including a eukaryotic microbial cell, including a yeast and a fungus, and a prokaryotic microbial cell including a bacteria.

The second determinant may also be a mammalian virus, including a human virus and  
15 an animal virus, a plant virus, a microbial virus, including a eukaryotic microbial virus, including a yeast virus and a fungal virus, and a prokaryotic microbial virus including a bacteriophage.

In one embodiment the substratum is porous and preferably a membrane. The flux of  
20 water through said material is preferably substantially unchanged as compared to the flux of water through said porous substratum. In another embodiment the substratum is non-porous and/or substantially non-penetrable to water.

There is also provided a material for use in a method of controlling cellular growth  
25 and/or cellular proliferation and/or cellular differentiation ex vivo, or a method of separating and/or isolating biological material ex vivo, or a method of producing a biohybrid organ ex vivo.

In another embodiment there is provided a material for use in a diagnostic method  
30 carried out on the human or animal body, or for use in a method of therapy carried out on the human or animal body, or for use in a method of surgery carried out on the human or animal body.

There is also provided a material for use in a method of producing a biohybrid organ in vivo, and a material for use as a carrier for in vivo delivery of a medicament to a human or animal body in need of said medicament. In another embodiment there is provided a material for use in a method of controlling cellular growth and/or cellular proliferation and/or cellular differentiation in vivo, and a material for use in a method of separating and/or isolating biological material in vivo.

In another aspect there is provided a composition comprising the material according to the invention and a physiologically acceptable carrier. The invention also pertains to a pharmaceutical composition comprising the material according to the invention or the composition as defined herein and a pharmaceutically active ingredient and optionally a pharmaceutically active carrier.

The pharmaceutically active compound is preferably selected from the group consisting of enzymes, hormones, cytokines, colony stimulating factors, vaccine antigens, antibodies, clotting factors, regulatory proteins, transcription factors, receptors, structural proteins, angiogenesis factors, human growth hormone, Factor VIII, Factor IX, erythropoietin, insulin, alpha-1 antitrypsin, calcitonin, glucocerebrosidase, low density lipoprotein (LDL) receptor, IL-2 receptor, globin, immunoglobulin, catalytic antibodies, the interleukins, insulin-like growth factor 1 (IGF-1), parathyroid hormone (PTH), leptin, the interferons, the nerve growth factors, basic fibroblast growth factor (bFGF), transforming growth factor (TGF), transforming growth factor-beta (TGF-beta), acidic FGF (aFGF), epidermal growth factor (EGF), endothelial cell growth factor, platelet derived growth factor (PDGF), transforming growth factors, endothelial cell stimulating angiogenesis factor (ESAF), angiogenin, tissue plasminogen activator (t-PA), granulocyte colony stimulating factor (G-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF).

There is also provided the use of the material or the composition or the pharmaceutical composition according to the invention in a method of therapy carried out on the human or animal body, a method of surgery carried out on the human or animal body, or a diagnostic method carried out on the human or animal body.

In another embodiment there is provided the use of the material or the composition or the pharmaceutical composition in a method of producing a biohybrid organ in vivo, or as a carrier for in vivo delivery of a medicament to a human or animal body in need of said medicament.

5

The invention also pertains to the use of the material or the composition or the pharmaceutical composition in a method of controlling cellular growth and/or cellular proliferation and/or cellular differentiation in vivo, or use of the material in a method of separating and/or isolating biological material in vivo, or use of the material in a method of controlling cellular growth and/or cellular proliferation and/or cellular differentiation ex vivo, or use of the material in a method of separating and/or isolating biological material ex vivo, or use of the material in a method of producing a biohybrid organ ex vivo, and the use of the material in the manufacture of an implantable organ or part thereof.

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The material according to the invention may also be used as a carrier for a pharmaceutically active ingredient or a pharmaceutical composition.

There is also provided a method of controlling cellular growth and/or cellular proliferation and/or cellular differentiation ex vivo, said method comprising the steps of contacting a cell with the material or the composition or the pharmaceutical composition according to the invention, and incubating said cell and said material under conditions allowing said cell to grow and/or proliferate and/or differentiate.

25 The invention also pertains to a method of separating and/or isolating biological material ex vivo, said method comprising the steps of contacting said biological material to be separated and/or isolated with the material or the composition or the pharmaceutical composition according to the invention, and incubating said biological material and said material under conditions that allow separation and/or isolation.

30

There is also provided a method of producing a biohybrid organ ex vivo, said method comprising the steps of contacting biohybrid organ cells with the material or the composition or the pharmaceutical composition according to the invention, and

incubating said biohybrid organ cells under conditions allowing the production of said biohybrid organ.

5 The invention also pertains to the following methods in particularly preferred embodiments:

10 Method of therapy carried out on the human or animal body, said method comprising the step of contacting said body with the material or the composition or the pharmaceutical composition according to the invention.

Method of surgery carried out on the human or animal body, said method comprising the step of contacting said body the material or the composition or the pharmaceutical composition according to the invention.

15 Method of diagnosis carried out on the human or animal body, said method comprising the steps of contacting said body with the material or the composition or the pharmaceutical composition according to the invention, and detecting a signal generated directly or indirectly by said material.

20 Method of controlling cellular growth and/or cellular proliferation and/or cellular differentiation in vivo, said method comprising the steps of contacting a cell with the material or the composition or the pharmaceutical composition according to the invention, and incubating said cell and said material under conditions allowing said cell to grow and/or proliferate and/or differentiate.

25 Method of separating and/or isolating biological material in vivo, said method comprising the steps of contacting said biological material to be separated and/or isolated with the material or the composition or the pharmaceutical composition according to the invention, and incubating said biological material and said material under conditions that allow separation and/or isolation.

30 Method of producing a biohybrid organ in vivo, said method comprising the steps of contacting biohybrid organ cells with the material or the composition or the

pharmaceutical composition according to the invention, and incubating said biohybrid organ cells under conditions allowing the production of said biohybrid organ.

5 Method of in vivo delivery of a medicament to a human or animal body in need of said medicament, said method comprising the steps of contacting said body with the pharmaceutical composition according to the invention and incubating said body contacted by said pharmaceutical composition under conditions allowing delivery of said medicament.

10 Additionally preferred embodiments of the invention are illustrated herein below. US 5,201,715 incorporated herein by reference relates to a target object having a characteristic ultrasonic signature for implantation beneath the skin. The object, when placed within an implanted injection port enables ultrasonic echographic discrimination of the target from surrounding tissues. The object enables one to locate  
15 the position of the object beneath the skin by non-invasive ultrasonic echography. The signature comprises reflections of ultrasonic waves from the object.

Accordingly, one embodiment of the present invention relates to a device for implantation beneath the skin capable of being located by non-invasive ultrasonic  
20 means at least when implanted. The device comprises a target comprising a biocompatible material according to the present invention comprising at least one and preferably a plurality of ultrasonically reflective surfaces, said at least one or a combination of said plurality of ultrasonically reflective surfaces providing a characteristic ultrasonic echographic signature. The biocompatible material preferably  
25 has an acoustical velocity which is different from the acoustical velocity of human tissue, and the object optionally further comprises a laminate structure consisting of substantially planar layers of bonded together biocompatible materials according to the present invention. The object preferably comprises a unitary structure.

30 US 5,976,780 incorporated herein by reference relates to a macroencapsulation device for somatic cells. Accordingly, the present invention in one embodiment relates to a transplantation or implantation device comprising

i) a hollow fiber comprising a material according to the present invention and having ends and a fiber wall with a porosity which selectively allows nutritional, gaseous, and metabolic substances to pass therethrough and which only allows passage of substances having a molecular weight less than about 30,000 Daltons, and

5

ii) a mixture of viable cells, preferably somatic, mammalian cells, and alginate gel suspended within said fiber.

Also provided is a device wherein said wall is devoid of macrovoids and has a porosity which prevents donor antigens and cytokines from passing through said wall. The device preferably comprises a fiber comprising a material according to the present invention capable of inhibiting complement activation. The somatic cells are preferably selected from the group consisting of neural, endocrine and hepatic cells, and said cells are preferably free from passenger leukocytes.

15

Also provided is a transplantation or implantation device comprising

i) a hollow fiber comprising a material according to the present invention and having ends and a fiber wall with a porosity which selectively allows nutritional, gaseous, and metabolic substances to pass therethrough and

20

ii) a mixture of viable cells, preferably viable, somatic, mammalian cells, and alginate gel suspended within said fiber.

The alginate optionally comprises ultrapurified alginate which is substantially free of divalent metal toxins and comprises (i) an endotoxin content of preferably less than 750 EU/g, (ii) a protein content of preferably less than 0.2%, and (iii) a G monomer, dimer and trimer content of preferably greater than 60%.

25

US 4,624,669 incorporated herein by reference relates to a corneal inlay for implant within the cornea and of a material such as polysulfone, wherein the inlay comprises a plurality of pores facilitating the passage of nutrients and fluids from the bottom

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surface layer of the cornea to the top surface layer of the cornea. Accordingly, one embodiment of the present invention pertains to a corneal inlay comprising:

5 i) an optic lens comprising a material according to the present invention for implantation within the cornea; and,

10 ii) a plurality of holes having a diameter of from 0.001 mm to 0.1 mm, said holes extending from a bottom surface to a top surface so as to allow for passage of nutrients through the cornea.

Also provided is a corneal inlay comprising:

15 i) an optic lens comprising a material according to the present invention for implant within the cornea; and,

ii) a plurality of slits, having a maximum width of from 0.01 mm to 0.05 mm, and a maximum length of from 0.05 mm to 1.0 mm, said slits extending from a bottom surface to a top surface so as to allow for passage through the cornea.

20 US 5,213,721 incorporated herein by reference relates to a porous device comprising a plurality of holes arranged in a predetermined, geometrical configuration. The holes are derived by means of a procedure of repetitive drawing. Prior to the first drawing operation, each of the holes is filled with a material which is soluble to a certain chemical, yet drawable along with the base material. Dependent upon the extent of  
25 drawing, a porous device is provided which includes holes of a significantly reduced cross-sectional area.

Accordingly, there is provided a device comprising a material according to the present invention for use as either a scaffold, a contact lens, an intracorneal inlay, an  
30 intraocular lens, a medical filter, or a similar structure with small holes. Accordingly, there is provided a scaffold or an optic device such as a contact lens comprising a material according to the present invention.

US 5,965,125 incorporated herein by reference relates to an implantable device having a body of matrix material made up of insoluble collagen fibrils, and disposed therewithin i) a plurality of vertebrate cells; and ii) a plurality of microspheres including microspheres consisting primarily of polysulfone.

5

Accordingly, the present invention in one embodiment relates to a composition comprising a body of matrix material, preferably a matrix material comprising insoluble collagen fibrils, and embedded within the body of said matrix material

10 i) a plurality of cultured cells, preferably vertebrate cells, even more preferably genetically engineered vertebrate cells, wherein said cells are capable of expressing a medically useful biologically active compound including a polypeptide; and

15 ii) a plurality of microspheres, wherein at least part of said microspheres comprises a material according to the present invention.

The cultured vertebrate cells are preferably selected from the group consisting of adipocytes, astrocytes, cardiac muscle cells, chondrocytes, endothelial cells, epithelial cells, fibroblasts, gangliocytes, glandular cells, glial cells, hematopoietic cells, 20 hepatocytes, keratinocytes, myoblasts, neural cells, osteoblasts, pancreatic beta cells, renal cells, smooth muscle cells, striated muscle cells, and precursors of any of the above.

It is preferred that the cultured vertebrate cells are transfected cells, preferably 25 transfected human cells comprising exogenous DNA encoding a medically useful biologically active compound including a polypeptide. The cultured vertebrate cells are preferably transfected cells containing exogenous DNA which includes a regulatory sequence that activates expression of a gene encoding said medically useful biologically active compound, preferably a polypeptide, wherein said gene is 30 endogenous to said vertebrate cells both prior to and after they are transfected.

The biologically active compound, preferably a polypeptide, is preferably selected from the group consisting of enzymes, hormones, cytokines, colony stimulating



factors, vaccine antigens, antibodies, clotting factors, regulatory proteins, transcription factors, receptors, structural proteins, angiogenesis factors, human growth hormone, Factor VIII, Factor IX, erythropoietin, insulin, alpha-1 antitrypsin, calcitonin, glucocerebrosidase, low density lipoprotein (LDL) receptor, IL-2 receptor, globin, immunoglobulin, catalytic antibodies, the interleukins, insulin-like growth factor 1 (IGF-1), parathyroid hormone (PTH), leptin, the interferons, the nerve growth factors, basic fibroblast growth factor (bFGF), transforming growth factor (TGF), transforming growth factor-beta (TGF-beta), acidic FGF (aFGF), epidermal growth factor (EGF), endothelial cell growth factor, platelet derived growth factor (PDGF), transforming growth factors, endothelial cell stimulating angiogenesis factor (ESAF), angiogenin, tissue plasminogen activator (t-PA), granulocyte colony stimulating factor (G-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF).

In a preferred embodiment, the biologically active compound, preferably in the form of a medically useful polypeptide, is administered to a patient by shunting a portion of the patient's blood so that the polypeptide secreted by the cells in the hybrid matrix mixes with the blood. Generally, any suitable method known to those of skill in the art can be used or adapted to accommodate the matrix of the invention. For example, blood shunted into a device which contains a perm-selective membrane surrounding a matrix comprising a material according to the present invention will result in the delivery of a therapeutic product of the matrix to the blood. A device similar to an artificial pancreas (Sullivan et al., Science 252:718-721, 1991) may be used for this purpose.

In another preferred embodiment, a hybrid matrix comprising a material according to the present invention is a means for producing a polypeptide in vitro. The method includes the steps of placing the hybrid matrix comprising a material according to the present invention under conditions whereby the cells in the matrix express and secrete a polypeptide of interest; contacting the matrix with a predetermined liquid such that the cells secrete the polypeptide into said liquid; and obtaining the polypeptide from the liquid, e.g., by standard purification techniques appropriate for the given polypeptide.

In one preferred embodiment, the matrix comprising a material according to the present invention is anchored to a surface and is bathed by the liquid; alternatively, the matrix floats freely in the liquid. Cells embedded in the hybrid matrix preferably function at a high level in a relatively confined space. Furthermore, the first step in purification of e.g. an expressed polypeptide (removal of the cells from the medium) is considerably more efficient with the matrices according to the present invention than with most standard methods of cell culture.

US 5,676,924 incorporated herein by reference relates to a method of determining the effectiveness of a cancer treatment by sealing tumor cells in segments of semipermeable membrane hollow fibers, implanting the sealed fiber segments in a mammal, treating the mammal with a cancer treatment, and evaluating the effect of the cancer treatment on the cells in the hollow fiber segments.

Accordingly, the present invention in one embodiment relates to a method of determining the effectiveness of a cancer treatment, said method comprising the steps of,

i) providing elongated segments of semipermeable membrane hollow fibers comprising a material according to the present invention and having a pore size effective to permit passage of nutrients, wherein said pore size excludes components of the immune system of a mammal, wherein said components are capable of inducing tissue rejection,

ii) placing by means of inserting said tumor cells into said hollow fibers, and sealing the ends of said hollow fibers,

iii) implanting said sealed hollow fibers intraperitoneally or subcutaneously into a non-human mammal,

iv) administering the cancer treatment, and

v) monitoring the effectiveness of said treatment on the cells in said implanted hollow fibers.

5 The mammal is preferably immunocompetent, such as a rat, and the tumor cells are preferably leukemic cells, preferably autologous leukemic cells or tumor cells obtained from a tumor of a patient. The tumor cells by be in suspension or in the form of a tissue explant.

10 US 5,830,708 incorporated herein by reference relates to methods for producing naturally secreted human extracellular matrix material and compositions containing this extracellular matrix material. The method includes culturing extracellular matrix-secreting human cells on a biocompatible, three-dimensional framework in vitro.

15 Accordingly, the present invention in one embodiment relates to a method for the production of human, naturally secreted extracellular matrix material, said method comprising the steps of:

20 i) providing a) a living tissue, optionally tissue prepared in vitro, preferably by culturing living tissue comprising human stromal cells such as fibroblasts, and b) connective tissue proteins naturally secreted by the living tissue, said connective tissue being attached to and substantially enveloping a material according to the present invention;

25 ii) killing the cells in the living tissue; and

iii) removing the killed cells and any cellular contents from the material according to the present invention, and

30 iv) collecting the extracellular matrix material deposited on the framework.

The method optionally further comprises the step of processing the collected extracellular matrix material by homogenizing, cross-linking, or suspending the extracellular matrix material in a physiological acceptable carrier.

The stromal cells, preferably fibroblasts, of the living stromal tissue are cells found in loose connective tissue or bone marrow, and preferably endothelial cells, pericytes, macrophages, monocytes, leukocytes, plasma cells, mast cells or adipocytes.

5

In one embodiment of this aspect of the invention there is provided an injectable material for soft tissue augmentation and related methods for use and manufacture of such materials, which overcome the shortcomings of state of the art bovine injectable collagen and similar injectable materials. The injectable materials according to the present invention comprise naturally secreted extracellular matrix preparations as well as preparations derived from naturally secreted extracellular matrix. These preparations are biocompatible, biodegradable and are capable of promoting connective tissue deposition, angiogenesis, reepithelialization and fibroplasia, which is useful in the repair of skin and other tissue defects. These extracellular matrix preparations may be used to repair tissue defects by injection at the site of the defect.

In another embodiment of the present invention, the preparations can be used in highly improved systems for in vitro tissue culture. Naturally secreted extracellular matrix coated three-dimensional frameworks comprising a material according to the present invention can be used to culture cells which require attachment to a support in order to grow, but do not attach to conventional tissue culture vessels. In addition to culturing cells on a coated framework, the extracellular matrix secreted by the cells onto the framework can be collected and used to coat vessels for use in tissue culture. The extracellular matrix, acting as a base substrate, may allow cells normally unable to attach to conventional tissue culture dish base substrates to attach and subsequently grow.

Yet another embodiment of the present invention is directed to a novel method for determining the ability for cellular taxis of a particular cell. The method involves inoculating one end of a native extracellular matrix coated three-dimensional framework comprising a material according to the present invention with the cell type in question, and over time measure the distance traversed across the framework by the cell. Because the extracellular matrix is secreted naturally by the cells onto the

framework, it is an excellent in vitro equivalent of extracellular matrix found in the body. Such an assay, for example, may inform whether isolated tumor cells are metastatic or whether certain immune cells can migrate across or even chemotact across the framework, thus, indicating that the cell has such cellular taxis ability.

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In another aspect of the present invention there is provided a method for producing the material according to the invention, said method comprising the steps of providing a substratum having a second contact angle, and contacting said substratum with a composition comprising a plurality of macromolecules. The method preferably  
10 pertains to the production of a material as described herein above. The substratum preferably comprises a hydrophobic polymer and said substratum may be pretreated prior to being contacted by said macromolecule. The pretreatment is effective in increasing the wettability of said substratum.

15 The macromolecule according to the method comprises a hydrophilic polymer, preferably a latently reactive polymer. The macromolecule preferably has a MW of more than 400 Da. The macromolecule comprises a conjugate comprising a likable head group, a linker group, a polymer chain, and a functional end group. The head group is preferably a photo-reactive aryl azide head group.

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The macromolecule may optionally comprise a modifying agent, preferably a modifying agent capable of contacting said substratum and forming a self assembled monolayer.

25 According to the method for producing the material according to the invention, said method may comprising the further step of contacting said material with a first determinant comprising a biologically active compound. The biologically active compound is preferably a polypeptide, an antibody, a polyclonal antibody, a monoclonal antibody, an immunogenic determinant, an antigenic determinant, a  
30 receptor, a receptor binding protein, an interleukine, a cytokine, a cellular differentiation factor, a cellular growth factor, or an antagonist to a receptor. The biologically active compound may be membrane associated and/or an extracellular

matrix polypeptide natively produced by a microbial cell, a plant cell or a mammalian cell.

5 According to the method of the invention, a further step of contacting said material with a second determinant comprising a biological entity may also be included. The biological entity comprises a cell or a virus, or a part thereof, and said cell, or part thereof, is preferably selected from the group consisting of a mammalian cell, including a human cell and an animal cell, a plant cell, a microbial cell, including a eukaryotic microbial cell, including a yeast and a fungus, and a prokaryotic microbial  
10 cell including a bacteria. When being a virus, or part thereof, said virus is preferably selected from a mammalian virus, including a human virus and an animal virus, a plant virus, a microbial virus, including a eukaryotic microbial virus, including a yeast virus and a fungal virus, and a prokaryotic microbial virus including a bacteriophage. Accordingly, the biological entity as defined herein preferably comprises a  
15 polypeptide, or a part thereof, a nucleic acid moiety, a carbohydrate moiety, and a lipid moiety, including any combination thereof. The biological entity may also comprise an antibody, a polyclonal antibody, a monoclonal antibody, an immunogenic determinant, an antigenic determinant, a receptor, a receptor binding protein, an interleukine, a cytokine, a differentiation factor, a growth factor, or an antagonist to  
20 the receptor.

The method of producing a material according to the invention relates in one preferred embodiment to a modification of a method described in U.S. Patent No. 5,741,551 (to Guire). Accordingly, the novel biomaterial surface layer is in one preferred  
25 embodiment generated by a two-step process using e.g. macromolecular amphiphiles with latent (photo) reactivity. Consequently, in a first step, amphiphilic macromolecules are allowed to adsorb to a suitable polymer substratum. The latent-reactive head-group will bring the amphiphils into reactive contact with the surface of the substratum. The hydrophilic main-body of the amphiphilic macromolecules  
30 exhibits a pronounced excluded volume leading to a lateral pattern of uniformly "self-assembled", adsorbed amphiphilic macromolecules. As described above, layer density and pattern depend on e.g. the amphiphilic character of the macromolecule such as e.g. chain length and/or degree of branching, the polymer substratum, as well as the

solution conditions (e.g. concentration, solvent, salt, temperature). As a consequence, the interface properties will be adjustable by altering the molecular characteristics of both the polymer substratum and the macromolecule. Similar or at least substantially similar monolayer structures are attainable on even quite different substrata by adjusting e.g. macromolecular properties or solution conditions. Amphiphil adsorption can readily be monitored by known surface physico-chemical methods such as e.g. ellipsometry or contact angle (CA) measurements.

In a second step, excess of macromolecules is removed and the latently reactive head-groups are activated. The activation results in the formation of a covalent bond formation between the macromolecule and the surface of the polymer substratum. Activation is preferably achieved by using electromagnetic radiation in the UV or Vis light range.

In a preferred embodiment, the method of producing a material according to the present invention is practiced with a macromolecule comprising a hydrophilic polymer, the hydrophilic polymer preferably being poly(ethylene glycol). The macromolecule preferably has a MW of more than 400 Da. The macromolecule further comprises a conjugate comprising a linkable head group, a linker group, a polymer chain, and a functional end group. The head group preferably is a photo-reactive aryl azide head group. In this preferred embodiment no irradiation is applied to the substratum, being contacted with said macromolecule, which could activate the latently reactive head group forming a covalent bond between the substratum and said macromolecule. Without being bound by theory it is believed, that the macromolecules contacting said substratum are anchored/immobilized to the underlying substratum by hydrophobic interactions and/or entanglement of the headgroup/guiding group and the hydrophobic substratum.

In one preferred embodiment, the method according to the present invention is practiced on a substratum that has not been pretreated. Substrata such as solid surfaces may be pre-washed to remove surface contamination and may be modified as desired to affect solvophilic characteristics without adding functional groups that are involved in covalent bond formation with e.g. latent-reactive groups. For example, polystyrene

surfaces may be washed and then exposed to hydroxyl ions in known water vapour plasma contact procedures so as to add hydroxyl groups to the substratum surface solely for the purpose of rendering the surface more readily wetted by aqueous solutions, the hydroxyl groups not being involved in subsequent covalent bond formation with the surface upon latent reactive group activation. Avoidance of pretreatment steps, defined in the definitions, leads not only to important processing economies but also avoids technical problems associated with the attachment of bond-forming reactive groups to surfaces at uniform loading densities.

### Examples

The following examples are illustrative of the present invention and will explain the invention in a non-limiting way.

#### Example 1

##### Synthesis of $\alpha$ -4-azidobenzoyl $\omega$ -methoxy poly(ethylene glycol)s (ABMPEG)

The synthesis of photo-reactive ABMPEG 5 kDa is described. ABMPEG of different MWs (2, 5, and 10 kDa) were employed as modifying agents in all following examples, all being synthesized as described in this example.

##### 1. Procedure

4-Azidobenzoic acid is prepared from 4-aminobenzoic-acid which is diazotized with sodium nitrate.<sup>[39,40]</sup> The carboxylic acid is converted into the 4-azido benzoyl chloride with thionyl chloride.<sup>[39,40]</sup> 0.23 g (1.875 mmol) of dimethylaminopyridine (DMAP) in 10 ml dry methylene chloride is mixed with 0.17 ml (1.250 mmol) triethylamine (TEA). The solution is transferred into a 250 ml three neck roundbottom flask. After cooling down to 0°C, 0.57 g (3.125 mmol) 4-azido benzoyl chloride in 10 ml CH<sub>2</sub>Cl<sub>2</sub> is added forming a yellow dispersion. 6.25 g (1.5 mmol) MPEG 5 kDa in 50 ml dry CH<sub>2</sub>Cl<sub>2</sub> is added dropwise during 1 hour under dry nitrogen, after which the temperature is allowed to rise to room temperature. The reaction is continued with stirring overnight. The solution is filtered, and ABMPEG is precipitated in cold



diethylether. The product is purified by two further precipitations from  $\text{CH}_2\text{Cl}_2$ /diethylether and dried in vacuum. Yield: 4.83 g (74 %).

## Example 2

### 5 Adsorption characteristics/kinetics of ABMPEG 5 kDa and MPEG 5 kDa to a polysulfone surface monitored by ellipsometry

Ellipsometry is a very sensitive technique for the determination of adsorption kinetics to optically smooth surfaces. For better resolution, transparent polysulfone (PSf) films  
10 were spin-coated onto polished silicon wafers, and thus the reflecting properties of the underlying silicon were exploited.

#### *1. Preparation of PSf surfaces*

Hydrophilic silicon slides: Silica surfaces are prepared from polished silicon wafers  
15 which are thermally oxidized in pure and saturated oxygen followed by annealing and cooling under argon flow to yield an oxide layer of about 30 nm. Wafers are cut into rectangular slides (10-14 mm x 20-30 mm), thoroughly cleaned with detergent, etched for 15 min in a freshly mixed 3:1 (v:v) sulfuric acid (96 %): hydrogen peroxide (30 %) solution, thoroughly rinsed, stabilized for 2 hours and rinsed again with/in ultrapure  
20 water. Slides are dried free of dust for two hours at 120°C. This procedure results in surfaces dense in silanol groups with a contact angle of less than 10°.

Hydrophobic silicon slides: In order to yield hydrophobic surfaces, previously prepared hydrophilic silicon slides are silanised in air saturated with hexamethyldisilazane (HMDS) at approx. 110°C. Excess HMDS is rinsed away with  
25 ultrapure  $\text{H}_2\text{O}$ . Slides are dried free of dust at room temperature.

PSf-spin-coated hydrophobic silicon slides: The previously prepared hydrophobic silicon slides are spin-coated with a 3 % (w:w) PSf in 1,2-dichlorobenzene solution. Slides are completely wetted by the polymer solution and then spun for 10 sec at 500 rpm and consecutively for 50 sec at 5.000 rpm in order to attain a smooth polymer  
30 film. Coated slides are dried for at least 4 hours at vacuum at 60°C.

#### *2. Ellipsometry measurements*

ABMPEG 5 kDa and mono-methoxy-PEG MPEG 5 kDa adsorption out of aqueous solution to PSf spin-coated HMDS-treated silicon slides is monitored *in situ* using an automated Rudolph Thin Film ellipsometer, type 43603-200E, equipped with a thermostated quartz cuvette.<sup>[38]</sup> Spin-coated slides are stabilized in 4.5 ml water for at least 15 min or until constant polarizer and analyzer signals are obtained. 0.5 ml of concentrated aqueous ABMPEG 5 kDa /MPEG 5 kDa solution is added yielding 5 ml solution at defined concentration. A magnetic stirrer is activated for 30 sec upon addition of the ABMPEG 5 kDa /MPEG 5 kDa concentrate in order to homogenize the solution. Polarizer and analyzer data is collected until apparent equilibrium is reached. From the attained data, it is possible to calculate thickness and refractive index of an adsorbed layer and/or its mass.<sup>[41]</sup> Adsorption data is calculated for approximated values of the partial specific volume and the ratio between the molar weight and the molar refractivity for both ABMPEG 5 kDa and MPEG 5 kDa respectively applying the same values for both species. Results for the calculated adsorbed mass are represented in arbitrary units as only approximated values of the partial specific volume and molar refractivity of ABMPEG 5 kDa and MPEG 5 kDa were at hand.

### 3. Results

Fig.10 depicts adsorption kinetics monitored by ellipsometry for ABMPEG 5 kDa and MPEG 5 kDa respectively. Enhanced adsorption (factor = 3.5) and prolonged equilibrium times (> 2h) are observed for ABMPEG 5 kDa when compared with MPEG 5 kDa. The pronounced difference in the adsorptive characteristics of the two materials indicates a strong affinity between the hydrophobic (aromatic) head-group of ABMPEG 5 kDa and the hydrophobic PSf surface. This affinity leads to an oriented layer, where the headgroup is in close contact with the underlying substratum and thus very well positioned to be effectively grafted through photo-activation. Furthermore, flushing with water (20 ml/min) does not effect the adsorbed amount, i.e. no desorption, neither of ABMPEG 5 kDa nor of MPEG 5 kDa, takes place. A similar behavior is to be expected also for ABMPEG of other MW, e.g. 2, or 10 kDa. In conclusion, this example illustrates how the photo-reactive headgroup of ABMPEG enhances the attractive interactions with a hydrophobic interface leading to increased adsorption in comparison to the non-conjugated MPEG.

### Example 3

#### Controlling polymer surface hydrophilicity and heterogeneity through photo-grafting of ABMPEG

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PSf spin-coated films on glass coverslips were modified with ABMPEG 2, 5, and 10 kDa. Desired degrees of hydrophilicity and thus surface density of the different ABMPEG on PSf were attained by adjusting bulk ABMPEG concentrations during a first adsorptive step. Contact angles (CA) were used to monitor resulting changes. Mixtures of different ABMPEG were applied in order to attain intermediate surface characteristics. The effectiveness of the photoreactive grafting was evaluated for ABMPEG 10 kDa by removing non-grafted ABMPEG moities.

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##### *1. Preparation of polymer surfaces*

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Glass coverslips are cleaned with detergent, rinsed with ultrapure water, and etched for 15 min at approx.  $40 \pm 5$  °C in a freshly mixed 3:1 (v:v) sulfuric acid (96 %): hydrogen peroxide (30 %) solution. Coverslips are thoroughly rinsed, stabilized for 2 hours and rinsed again with/in ultrapure H<sub>2</sub>O. Slips are dried free of dust for two hours at 120°C. n-octadecyldimethylchlorosilane (ODDMS) is grafted to the cleaned coverslips by immersing them in a 2 % (w:w) ODDMS in n-hexane solution for 1 hour at room temperature. Coverslips are rinsed twice with n-hexane and three times with ethanol and air dried at room temperature. The ODDMS-treated coverslips are spin-coated with a 3 % (w:w) PSf in 1,2-dichlorobenzene solution. Coverslips are completely wetted by the polymer solution and then spun for 10 sec at 500 rpm and consecutively for 50 sec at 5.000 rounds per minute (rpm) in order to attain a smooth polymer film. Coated coverslips are dried for at least 4 hours at vacuum at 60°C.

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##### *2. ABMPEG grafting to polymer surface*

ABMPEG grafting includes the following two consecutive steps as illustrated in Fig.4.

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In a first adsorption step aqueous ABMPEG solution of different concentrations is placed on the PSf coated coverslip, covered and kept in the dark for at least 12 h but maximal 18 h. Thereafter coverslips are gently rinsed in ultrapure water, covered by water and immediately exposed to UV light for 1 min. For UV irradiation a 50 W high

pressure mercury lamp (ORIEL) equipped with a condenser is used. The UV rich light passes a high-pass glass filter with a cut off at 320 nm yielding an intensity of 30 mW/cm<sup>2</sup>. Certain indicated control surfaces are not exposed to UV irradiation. To remove non-covalently bond ABMPEG certain indicated sample surfaces were exposed over night to a 1:1 (v:v) water:isopropanol mixture (H<sub>2</sub>O/IP), thoroughly rinsed with the same mixture and with ultrapure water thereafter.

### 3. Contact angle (CA) measurements

As on modified and unmodified PSf coated coverslips are measured using the captive bubble method, where an air bubble is injected from a syringe with a stainless steel needle onto the inverted sample surfaces under water. The diameter of the contact area between the PSf film and the bubbles is always greater than 3 mm. While the needle remains inside the bubble, advancing and receding angle measurements are realized with a goniometer fitted with a tilting stage by stepwise withdrawing/adding air from/to the captured bubble. At least ten measurements of different bubbles on at least three different locations are averaged to yield one data.

### 4. Results

Fig.11 shows advancing and receding As of PSf spin-coats modified with different concentrations of ABMPEG 10 kDa. Surfaces were exposed to UV irradiation but not rinsed with (H<sub>2</sub>O/IP). Note that under the valid assumption that adsorbed ABMPEG layers are in the relevant time scales stable in aqueous environment, i.e. no desorption will take place (see results in Example 2), ABMPEG 10 kDa adsorption is monitored and not its chemical grafting. With rising ABMPEG 10 kDa bulk concentrations decreasing advancing and receding As are observed while CA-hysteresis increases in the applied concentration range. The results indicate that ABMPEG 10 kDa adsorption is highly controllable and reproducible. Desired degrees of hydrophilicity and thus surface density of ABMPEG 10 kDa are attained by adjusting bulk ABMPEG 10 kDa concentrations during adsorption.

Fig.12 shows As of surfaces which were modified applying ABMPEG of three different chain lengths, i.e. three different MWs: 2, 5, and 10 kDa (see also Fig.13). Again, surfaces were exposed to UV irradiation but not rinsed with H<sub>2</sub>O/IP thereafter. The same trend regarding degree and controllability of the attained hydrophilization of

the underlying PSf is observed for all different chain lengths, but differences in CA-hysteresis are observed. CA-hysteresis values are in general lower for shorter chain lengths, and a clear maximum is seen especially for the lowest MW ABMPEG in the applied concentration range. Thus longer chain lengths seem to induce more chemical and/or morphological heterogeneity manifested in increased CA-hysteresis.

Fig.14 shows receding As and CA-hysteresis of PSf surfaces which were modified with different mixtures of ABMPEG of two different chain lengths (ABMPEG 2 kDa and ABMPEG 10 kDa). Again, surfaces were exposed to UV irradiation but not rinsed with H<sub>2</sub>O/IP thereafter. Surfaces show a gradual change in surface properties. This result implies that mixtures of different ABMPEG and/or ABMPEG derivatives can be applied in order to attain/design intermediate surface characteristics.

Fig.15 shows receding As of PSf surfaces modified with different concentrations of ABMPEG 10 kDa. Samples were exposed to UV irradiation and the As measured before and after over night rinsing with H<sub>2</sub>O/IP. The data characterizes the efficiency of the photo-grafting process in dependence of applied ABMPEG concentration. For ABMPEG concentrations higher than 10 g/l the effectiveness of the photoreactive grafting diminishes rapidly manifested in the reversibility of the hydrophilization upon rinsing with H<sub>2</sub>O/IP. This indicates a decrease in head-group orientation towards the surface lowering chances for successful grafting. Increased solute-solute interactions at rising surface coverage might be responsible.

#### **Example 4**

##### **Assay of protein adsorption to modified PSf membranes**

Commercially available standard ultrafiltration membranes were modified at different degrees of modification with ABMPEG 5 kDa. Thereafter, the adsorptive properties of the membranes were evaluated by exposing them to a buffered solution of bovine serum albumine (BSA) and determining the adsorbed amount of BSA.

##### *1. Membrane modification*

Rinsed pieces of circular cut PSf ultrafiltration membrane (132.6 cm<sup>2</sup>, type GR61PP, DOW, Danmark) () were stabilized and cleaned from packaging liquids by permeating at least 6 liter of ultrapure water at 0.4 MPa for at least 1 h. The membrane was then cut

into circular membranes of 25 mm diameter and their skin-layer modified with ABMPEG 5 kDa as described in Example 3. After exposure to UV irradiation were membranes exposed to ultrasound for 5 min and thoroughly rinsed thereafter.

## 5      2. Protein adsorption

The skin-layer of unmodified or modified membranes was contacted for 2 hours with a 1 g/l BSA solution (0.15 molar phosphate buffer, pH=7, room temperature), flushed with buffer and dried at 60°C over night. Adsorbed amount of BSA is determined by its total hydrolysis and consecutive amino acid analysis.<sup>[42]</sup>

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## 3. Results

Fig.16 shows adsorbed amounts of BSA in dependence of the applied ABMPEG 5 kDa concentration. BSA adsorption decreases for increasing ABMPEG 5 kDa concentration. Maximum reduction in comparison to an unmodified reference membrane of about 70 % is attained for the highest applied ABMPEG 5 kDa concentration of 10 g/l.

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## Example 5

**Fibronectin (FN) adsorption to unmodified and ABMPEG 10 kDa modified PSf as measured by *in situ* ellipsometry**

As in Example 3, the reflecting properties of polished silicon were exploited to monitor FN adsorption to unmodified or modified spin-coated PSf films. Adsorption kinetics of FN to the differently modified surfaces are attained yielding information about the interfacial interactions of FN with the photo-grafted ABMPEG 10 kDa interfacial structure.

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## 25      1. Procedure

Fibronectin (FN) (human plasma, lyophilized, MW 440 kDa; Boehringer Mannheim, Germany) is reconstituted in phosphate-buffered saline (PBS; 5.8 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, pH = 7.4) containing 0.02 % (w/v) sodium azide giving a concentration of about 0.12 g/l. The instrumental setup and the measurement procedure are identical to the one described in Example 2.1 and 2.2 respectively. PSf

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films on silicon wafers, modified with ABMPEG 10 kDa at different concentrations as described in Example 3.1 are placed in the quartz cuvette and stabilized in 2.5 ml PBS buffer for at least 15 min or until constant polarizer and analyzer signals are obtained. 0.5 ml of the concentrated FN solution is added yielding 3 ml with a defined concentration of 0.02 g/l. The magnetic stirrer is activated for 2-3 sec upon addition of the protein concentrate in order to homogenize the solution. After 30 min the cuvette is flushed for 10 min with PBS buffer using preinstalled tubings and a flow rate of 20 ml/min. Even if plateau values are typically observed after 1-2 hours (or much longer), it is possible to describe protein – substratum interactions also already after 30 min.

In the calculations of the amount of protein adsorbed, the different layers, silicon support, silicon oxide, ODMS-layer, PSf-film, and tethered ABMPEG 10 kDa are treated as one optical unit with an effective refractive index. The molar refractivity of FN is calculated as the sum of the individual molar refractivities of all amino acids in FN using tabulated values<sup>[43]</sup> yielding a value of 3.99 g/ml. For the partial specific volume of FN the value 0.75 ml/g is used.

Fig.17 shows that all data curves follow the expected monotonic rise; FN desorption upon flushing is not observed. The adsorbed amount of FN decreases with higher degrees of ABMPEG 10 kDa surface functionalization and thus correlates qualitatively with the CA decrease as shown in Fig.11. Maximum adsorption of almost  $1.2 \mu\text{g}/\text{cm}^2$  is attained for both, unmodified PSf and PSf modified with the lowest ABMPEG 10 kDa concentration, i.e. 0.001 g/l. The adsorbed FN amount decreases by more than 60 % to  $0.45 \mu\text{g}/\text{cm}^2$  for an ABMPEG 10 kDa concentration of 10 g/l. As shown in Example 4, BSA adsorption to PSf UF membrane surfaces photo-grafted with ABMPEG 5 kDa and quantified by total hydrolysis and consecutive amino acid analysis of the adsorbed protein yielded very similar results: The relative reduction depending on the degree of functionalization correlates very well with the here presented results for FN.

**Example 6****Fibroblast adhesion to unmodified and ABMPEG 10 kDa modified PSf surfaces: overall cell morphology, number of adherent cells, and focal adhesion formation**

5 The number of adherent cells, the overall cell morphology, and the development of focal adhesions are good indications for the quality of interactions between cells and interfaces. Many previous studies have shown, that the more cells adhere, the more spread the cells are, and the more pronounced focal adhesions are formed, the better suited are the respective surfaces for the anchorage and proliferation of the  
10 investigated cells.

*1. Cells*

Human fibroblasts (HF) were obtained from fresh skin biopsy and used up to the 9th passage. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM)  
15 containing 10 % fetal bovine serum (FBS, Sigma Chemicals Co., St. Louis, MO, USA) in an humidified incubator with 5 % CO<sub>2</sub>. HF from nearly confluent cultures were harvested with 0.05 % trypsin/0.6 mM EDTA (Sigma), and trypsin was neutralized with FBS.

*2. Number of adherent HF and their morphology*

Adhesion of HF was carried out in 6-well tissue culture plates containing the unmodified and ABMPEG 10 kDa modified PSf coated glass slides. Experiments were performed without or with pre-coating of the surfaces with FBS (Sigma) for 30 min at 37°C. Approximately  $5 \cdot 10^5$  cells in DMEM were pipetted into each well  
25 and incubated for 2 h at 37°C in a humidified CO<sub>2</sub> incubator. The number of adherent cells and their morphology was studied and photographed directly from the wells with an inverted phase contrast microscope Telaval 31 (Carl Zeiss, Germany). The mean number of adherent cells was determined by evaluating approx. 30 different randomly chosen microscopic fields on each surface. Cell counts were normalized to: number of  
30 cells per area of microscopic field; the standard deviation was determined for each set of fields on a surface.

*3. Focal adhesions formation*



Focal adhesions of HF plated on non-precoated and serum-precoated substrata were visualized by immunofluorescence. Samples were processed as follows: Attached cells were fixed with paraformaldehyde (3 %) for 10 min and permeabilized with 0,2 % Triton X-100 for 5 min. To detect focal adhesions, samples were incubated for 30 min at 37°C with monoclonal anti vinculin antibody (Sigma Immunochemicals, St. Louis, MI, USA), followed by Cy3 conjugated goat anti mouse secondary antibody (Jackson Immuno Research, Inc. West Grove, PA, USA). Samples were mounted with Mowiol, and viewed and photographed with a inverted fluorescent microscope Axiovert 100 (Carl Zeiss, Germany).

#### 4. Results

Already shortly after plating cells (2 h), clear differences can be observed in dependence of the underlying substratum.

Fig.18 shows the overall cell morphology of adherent HF. A clear dependence between the amount of adherent cells (see also Fig.19) and their spreading and the employed ABMPEG 10 kDa concentrations can be seen in the phase-contrast pictures. PSf modified at intermediate concentrations of ABMPEG 10 kDa (0.001 – 0.01) shows increasing adherence and spreading of the plated HF-cells.

Focal adhesion formation on non-precoated substrata illustrated in Fig.20 demonstrates again significantly improved cell morphology and spreading on PSf surfaces modified with intermediate concentration of ABMPEG 10 kDa (0.001 g/l and 0.01 g/l), in comparison to unmodified PSf, or PSf modified with relatively high concentrations of ABMPEG 10 kDa (1 g/l and 10 g/l). Focal adhesion formation on serum-precoated substrata illustrated in Fig.21 represents the optimal focal adhesions formation on substrata modified with intermediate concentration of ABMPEG 10 kDa (0.001 g/l (B), and 0.01 g/l (C)). At 0.1 g/l (D), focal adhesions already start to disorganize and almost completely disappear at 10 g/l (F). An important observation is that the effect of ABMPEG density on focal adhesions formation is much more pronounced on serum coated ABMPEG surfaces (see Fig.20, and compare with Fig.21). Thus, modifying PSf with minute amounts of ABMPEG leads to much enhanced cell-substratum interactions.

**Example 7****Fibronectin matrix formation of fibroblasts adhering on unmodified and ABMPEG 10 kDa modified PSf surfaces**

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Fibronectin (FN) is an adhesive protein being essential for the adhesion/anchorage of cells to any kind of substratum. Shortly after contacting a suitable substratum, viable HF-cells will secrete FN and will form a FN matrix. The amount of secreted FN and the structure of the consecutively formed matrix can be used to evaluate the quality of cell-substratum interactions.

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*1. FN matrix formation*

Approximately  $5 \times 10^5$  HF in 3 ml medium containing 10 % FBS were incubated for 5 days in 6-well tissue culture plates (Falcon, Becton Dickinson & Company, New Jersey, USA) containing the PSf coated and photo-modified glass slides. At the end of the incubation cells were fixed with 3 % paraformaldehyde and FN matrix deposited on the different surfaces was visualized by immunofluorescence using a specific anti human FN matrix mouse monoclonal antibody (lot No. 0326, Immunotech SA, France), followed by Cy3-conjugated goat anti mouse secondary IgG1 antibody (Jackson Immuno Research, Inc. West Grove, PA, USA). Further investigations and photography were carried out with an inverted fluorescence microscope as above.

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*2. Results*

Fig.22 demonstrates maximal FN matrix formation of HF cultured on surfaces with moderate ABMPEG 10 kDa density. Note, that the secreted FN was also highly organized on these surfaces (see Fig.23).

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**Example 8****Proliferation of fibroblasts, liver cells, and endothelial cells on unmodified and ABMPEG 10 kDa modified PSf surfaces**

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Phase-contrast photographs were executed in order to characterize overall cell morphology and proliferation of human fibroblasts (HF), liver cells (C3A), and human

umbilical vein endothelial cells (HUVEC) on unmodified PSf as well as on ABMPEG 10 kDa modified PSf. Proliferation of HF was further characterized by semi-quantitative XTT and LDH assays which are established functional methods for cell proliferation. The XTT assay is based on the reductive cleavage of a water soluble tetrazolium salt by the dehydrogenase activity of intact mitochondria in the cells which can be quantitatively followed by a color change. The LDH assay monitors directly the activity of lactate dehydrogenase released by the cells.

### *1. Polymer surfaces*

Glass coverslips (15 x 15 and 18 x 18 mm<sup>2</sup>) and slides (26 x 76 mm<sup>2</sup>) were cleaned, hydrophobized, PSf spin-coated, and ABMPEG 10 kDa modified as described in Example 3.1 and 3.2. Surfaces were stored in 0.02% NaN<sub>3</sub> solution, and before plating with cells washed with distilled water and immersed into 70 % ethanol for 10 sec followed by air-drying under sterile conditions. Cover slips (15x15 mm<sup>2</sup>) were inserted into 12-well-plates, larger cover slips into 6-well-plates. Slides were compartmented by applying a Flexiperm silicon mask dividing the polymer surface into 8 wells.

### *2. Cells and proliferation studies*

HF were cultivated and harvested as described in Example 6.1. For HUVEC and C3A other culture media were employed (HUVEC: endothelial cell growth medium, C3A: MEM) but otherwise grown and harvested in the same way as HF. After centrifugation and resuspension the cell number was counted in a Neubauer counting chamber.

For cell proliferation studies the following cells and densities were applied:

	8-well-array	6-well-array	
seeding area (cm <sup>2</sup> )	0.88	9.08	cells/ cm <sup>2</sup>
HF	20,000/well		22,700
HUVEC		50,000/well	5,500
C3A		200,000/well	22,000

Cells were seeded into the wells and incubated at 37 °C and 5 % CO<sub>2</sub> up to 7 days. After 3, 5 and 7 days samples were inspected visually and their morphology / state was documented by phase-contrast photographs. After 1, 3 and 7 days XTT and LDH assays were performed.

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### 3. Results

For all three cell types the best growth conditions were observed on PSf modified with ABMPEG 10 kDa at concentrations ranging from 0.01 g/l to 0.1 g/l (see Fig.24 for HF, Fig.25 for HUVEC, and Fig.26 for C3A). Both, HF and C3A proliferated very well during the 7 day cultivation period and overgrew almost the whole substratum area for the named intermediate ABMPEG concentrations. HF were even found to grow in multilayers. The number of HUVEC was lower because of less initial seeding density (only 5,500 cells/cm<sup>2</sup> instead of more than 22,000 cells/cm<sup>2</sup> for HF and HUVEC).

The XTT and LDH assays performed for HF confirmed the observed trend (see Fig.27 and Fig.28), i.e. a maximum of proliferation for PSf surfaces modified at intermediate degrees of ABMPEG 10 kDa concentration. However, these assays show a much less pronounced maximum as compared with the phase contrast photographs. This was most likely due to boundary effects originating from the used Flexiperm silicon wells. Pronounced cellular adherence and proliferation was observed for the contact line of the silicon with the underlying PSf substratum.

### Example 9

#### **Focal adhesion formation of endothelial cells on unmodified and ABMPEG 10 kDa modified PSf**

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The development of focal adhesions is a measure for the quality of interactions between cells and interfaces (cf. Example 6).

#### 1. Cell cultivation

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The studied surfaces were pre-coated with FN (for details see Ref. [13]). HUVEC were cultivated and plated as described in Example 8. Immunofluorescence studies were carried out as described in Example 6.3 in order to visualize points of focal

adhesion between HUVEC and the underlying substratum. After incubation for 2 h, cells were inspected by phase-contrast microscopy and afterwards fixed with 3 % paraformaldehyde in PBS for 15 min. The further characterizations were performed as described in Example 6.3.

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## 2. Results

Fig.29 clearly shows the same dependence of the formation of focal adhesions on the degree of ABMPEG 10 kDa modification of the PSf substratum already seen before in the proliferation and adhesion studies. HUVEC plated on PSf substrata modified at intermediate concentrations of ABMPEG exhibit the highest number of focal adhesions and the best developed ones.

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### Example 10

**Ellipsometric evaluation of the interactions of proteins, adsorbed on different surfaces, with their respective antibodies.**

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The binding of antibodies to their respective antigens is only effective when both, the antigen as well as the antibody, are present in their native or biological active conformation. Antigen/antibody-binding usually represents a tight bond comprising a high dissociation constant. As discussed before, upon adsorption proteins often lose their conformational integrity and thus also their ability to bind to respective antibodies.<sup>[44]</sup> Several studies have characterized these changes in conformation/antibody-binding by monitoring either the release of bound antigen,<sup>[45,46]</sup> or the binding of the respective antibody to the previously adsorbed antigen e.g. by ellipsometry.<sup>[47,48]</sup>

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In this example we represent data which shows the increasing binding affinity of adsorbed proteins (antigens) in respect to their antibodies when these antigens were adsorbed to previously ABMPEG-modified interfaces. As a model system we choose bovine immuno globulin (BGG) and an enzyme-labeled anti-BGG(H+L), where the latter is an antibody directed towards the heavy and the light chains of BGG, i.e. towards four independent epitopes present on each BGG molecule. Furthermore, we

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applied human serum albumin (HSA) as blocking agent in order to cover remaining surface adsorption sites before exposing the anti-BGG to previously adsorbed BGG.

### *1. Preparation of surfaces.*

- 5 Hydrophilic, hydrophobic and PSf-spin-coated silicon slides were prepared as described in Example 2.1. PSf-spin-coated slides were grafted at a range of grafting densities with ABMPEG 10 kDa as described in Example 3.2.

### *2. Materials*

- 10 BGG-FITC: Fluorescein (FITC)-conjugated ChromPure BGG, (lot 001-090-003, Jackson ImmunoResearch Laboratories, Inc.; purified over a-BGG(H+L)-column); i.e. the Antigen, abbreviated with BGG
- a-BGG(H+L)-HRP: Horseredish peroxidase (HRP)-conjugated Goat Anti-Bovine BGG(H+L) from pooled antisera from goats hyperimmunized with BGG (Southern Biotechnology Associates, Inc.); i.e. the antibody, abbreviated with a-BGG
- 15 HSA: Human Serum Albumin (Centeon Pharma GmbH); i.e. the blocking agent

### *3. Ellipsometric determination of the consecutive adsorption of i) BGG as antigen, ii) HSA as blocking agent, and iii) a-BGG as respective antibody to BGG to unmodified and ABMPEG modified PSf-spin-coated silicon slides*

The instrumental set-up and the principal measurement procedure are described in Example 2.2. The differently modified PSf-spin-coated silicon slides were placed in the quartz cuvette and stabilized in 2.5 ml phosphate buffered saline (PBS; 154 mM NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, pH = 7.4) until constant polarizer and analyzer signals were obtained. The previously purified proteins, BGG, a-BGG, and HSA, were reestablished in PBS. At the start of each experiment (0 min), 0.5 ml of BGG-FITC solution were added to the cuvette yielding 3 ml with a defined concentration of 0.01 g/l. The contents of the cuvette was constantly stirred by a magnetic stirrer during all experiments. After 30 min of BGG adsorption the cuvette was flushed for 1 min with PBS using preinstalled tubings and a flow rate of 20 ml/min. Thereafter, the total volume in the cuvette was readjusted to 2.5 ml. After another minute (period

for signal stabilization), 0.5 ml of the concentrated HSA solution were added (at 32 min) yielding 3 ml with a defined concentration of 3 g/l. After 10 min of HSA adsorption the cuvette was flushed with PBS and the total volume readjusted as described above. At 44 min, 0.25 ml of the concentrated a-BGG solution were added yielding 2.75 ml with a defined concentration of 0.015 g/l. After 60 min of a-BGG adsorption (at 104 min) the cuvette was flushed with PBS buffer for 2 min and finally signal stabilization awaited for 1 min. At 107 min the experiment ended.

Polarizer and analyzer data were automatically collected during the whole period and the corresponding  $\Psi$  and  $\Delta$  values directly calculated. Relative changes in the calculated  $\Psi$  signal (the change in  $\Psi$  signal is proportional to the total mass adsorbed) during the periods of stabilization in PBS were used to compare adsorbed amounts of the different consecutively adsorbed proteins. All presented data is the arithmetic average of two independent experimental runs.

### *3. Different control experiments*

Consecutive adsorption of BGG, HSA and a-BGG, as described in the previous paragraph, was also performed on hydrophobic and hydrophilic silicon slides.

On some selected ABMPEG modified PSf-spin-coated silicon slides the first adsorption step, i.e. the adsorption of the antigen BGG, was not performed. However, adsorption of HSA and a-BGG was performed as described in the previous paragraph.

### *4. Results*

The monitored  $\Psi$  signal of two entire adsorption experiments, i.e. the consecutive adsorption of BGG, HSA and a-BGG, to unmodified PSf and PSf modified with 10 g/l ABMPEG, is shown in Fig.30.

For the unmodified PSf, the BGG adsorption kinetic levels off to an almost constant value of  $\Psi = 0.40$  after about 20 min. After 30 min, as indicated by the first arrow, PBS flushing started, followed by the addition of HSA (second arrow) intended to work as a blocking agent to cover residual surface area not covered by BGG. The applied concentration of HSA was 300 times higher than the applied BGG concentration, however, between the two PBS flushings (first and third arrow),  $\Psi$  rose only by 0.27 units, indicating that the polymer interface was already substantially saturated by BGG. Furthermore, the fast leveling off of the  $\Psi$ -signal upon addition of

HSA, a protein of smaller size and higher adhesiveness than BGG, indicates the efficient blocking of residual uncovered interface area. The addition of a-BGG (fourth arrow), again at a low concentration, followed by flushing (between fifth and sixth arrow) yielded however a substantial rise in the  $\Psi$ -signal (0.52 units). This rise is attributed to the high affinity antigen-antibody binding, yielding a second protein layer on top of the adsorbed BGG/HSA layer. The comparatively slow kinetics of the a-BGG binding is a further indication for a different mechanisms of binding of this second layer, i.e. a antigen-antibody binding vs. adsorptive binding.

For the PEG-modified PSf-ABMPEG 10g/l, the BGG adsorption proceeds much slower and to a far smaller extent (up to 0.035 units). The already present and covalently fixed ABMPEG reduce the available surface area and also reduce the speed of adsorption, due steric hindrance of ABMPEG moieties towards approaching BGG molecules. HSA, as mentioned a much smaller and more adhesive protein than BGG, is however less restrained of adsorbing in between the already present ABMPEG moieties, and thus adsorbing to a similar extent (0.33 units) as on PSf only covered by BGG (see above). The consecutive binding of a-BGG is comparatively smaller (0.15 units) than for the unmodified PSf (0.52 units). However, not the total amount of a-BGG bound to the surface characterizes the binding affinity of the previously adsorbed BGG, but the ratio between bound a-BGG and BGG. This ratio, however, increases in this experimental run from 1.2 for unmodified PSf to 4.3 for the PEG modified PSf-ABMPEG 10g/l indicating a much higher binding affinity and thus higher conformational integrity or biological activity of BGG when adsorbed to PEG-modified PSf.

Fig.31(a-c) shows the arithmetic mean of the relative rise in  $\Psi$ -signal for the consecutive adsorption of BGG, HSA, and a-BGG for all performed experiments on unmodified PSf (ref.) and ABMPEG-modified PSf. The error bars represent the standard deviation of the duplicated experimental runs. As expected, BGG adsorption decreases with increasing ABMPEG grafting density by approx. 95% from 0.5 to 0.03  $\Psi$ -units (Fig.31(a)). Consecutive adsorption of comparatively highly concentrated HSA yields a maximum for PSf modified with ABMPEG at a concentration of 1 g/l (Fig.31(b)). a-BGG adsorption decreases with increasing ABMPEG grafting density



by approx. 75% from 0.62 to 0.15  $\Psi$ -units (Fig.31(c)). Consequently rises the ratio between the adsorbed amount of a-BGG and previously adsorbed BGG with increasing ABMPEG grafting density from 1.25 for PSf (ref.) to 5.0 (the latter values being arithmetic means of two independent experimental runs) for PSf-ABMPEG-10g/l (Fig.31(d)).

Control experiments with hydrophobic wafers yielded similar results as observed for unmodified PSf (BGG =  $0.39 \pm 0.08$ , HSA =  $0.17 \pm 0.02$ , a-BGG =  $0.40 \pm 0.05$ ), the ratio between a-BGG and BGG being  $1.03 \pm 0.08$ , i.e. slightly lower than for PSf. Control experiments performed on hydrophilic wafers did not yield conclusive results as the total amount of adsorbed proteins was too low.

In order to verify the efficiency of the blocking agent, HSA, ABMPEG modified PSf slides (modified at 0.01 and 1.0 g/l ABMPEG) were directly exposed to HSA under the same conditions as in the previous adsorption experiments, i.e. for 10 min to PBS buffered HSA solution of 3 g/l. Consecutive exposure to a-BGG solution (following the above procedure, i.e. 0.015 g/l a-BGG, time of adsorption: 60 min) yielded for both surfaces a slight increase of the  $\Psi$ -signal (0.1 units). However, when comparing this increase with the increase obtained in the presence of previously adsorbed BGG (see Fig.31(c)), a much more pronounced a-BGG adsorption is observed, yielding on average an increase of 0.38 units, i.e. a four fold higher value.

Several conclusions can be drawn from this data:

- i) BGG adsorption to ABMPEG modified PSf surface decreases for higher degrees of ABMPEG surface modification (see Fig.31(a)), as also observed for FN and BSA in example 5. This confirms that, independent of the adsorbing protein (FN, BSA, or BGG), increasing ABMPEG grafting density decreases protein adsorption to these surfaces.
- ii) Under the assumptions that, a) HSA does effectively block the interface available for additional adsorption, and that b) a-BGG does not bind to a great extent to neither adsorbed HSA (as shown) nor grafted ABMPEG (can be assumed, see also Ref.[14] and [33]), an increase in the ratio of adsorbed a-BGG with respect to

adsorbed BGG can be interpreted as an increase of conformational integrity or biological activity of the adsorbed BGG.

## References

- [1] Williams, D.F. *Definitions in biomaterials*, Elsevier, Amsterdam (1987).
- [2] Saltzman, W.M., Cell interactions with polymers, in: *Principles of tissue engineering*, 1<sup>st</sup> ed., R. Lanza R.G. Landes Company, (1997), 225ff.
- 5 [3] Colton, C.K., Implantable biohybrid artificial organs, *Cell Transplantation* **4**, 415 (1995).
- [4] Andrade, J.D., Hlady, V. Protein Adsorption and Materials Biocompatibility: A Tutorial Review and Suggested Hypotheses, *Adv.Polym.Sci.* **79**, 1 (1986).
- [5] Norde, W., Lyklema, J., Why proteins prefer interfaces,  
10 *J.Biomater.Sci.Polymer Edn.* **2**, 183 (1991).
- [6] Grinnell, F., Cellular adhesiveness and extracellular substrata, *Int. Rev. Cytol.* **53**, 65 (1978).
- [7] Grinnell, F., Fibronectin adsorption on material surfaces, in: *Blood in contact with natural and artificial surfaces*, 1<sup>st</sup> ed., E.F. Leonard Ann.N.Y.A.Sci.  
15 New York (1987), 280ff.
- [8] Grinnell, F., Feld, M., Fibronectin adsorption on hydrophilic and hydrophobic surfaces detected by antibody binding and analyzed during cell adhesion in serum containing medium, *J.Biol.Chem.* **257**, 4888 (1982).
- [9] Hynes, R.O., Integrins: A family of cell surface receptors, *Cell* **48**, 549  
20 (1987).
- [10] Hynes, R.O., Integrins: Versatility, modulation and signaling, *Cell* **69**, 11 (1992).
- [12] Juliano, D.J., Saavedra, S.S., Truskey, G.A., Effect of the conformation and orientation of adsorbed fibronectin on endothelial cell spreading and the  
25 strength of adhesion, *J.Biomed.Mat.Res.* **27**, 1103 (1993).
- [13] Groth, T., Zlatanov, I., Altankov, G., Adhesion of human peripheral lymphocytes on biomaterials preadsorbed with fibronectin and vitronectin, *J.Biomater.Sci.Polymer Edn.* **6**, 729 (1994).
- [14] Lee, J.H., Lee, J.W., Khang, G., Lee, H.B., Interactions of cells on chargeable  
30 functional group gradient surfaces, *Biomaterials* **18**, 351 (1997).
- [15] Fabrizio-Homan, D.J., Cooper, S.L., A comparison of the adsorption of three adhesive proteins to biomaterial surfaces, *J.Biomater.Sci.Polymer Edn.* **3**, 27 (1991).

- [16] Massia, S.P., Hubbell, J.A., Covalent surface immobilization of Arg-Gly-Asp- and Tyre-Ile-Gly-Ser-Arg-containing peptides to well defined cell adhesive aubstrates, *Analy.Biochem.* **187**, 155 (1990).
- 5 [17] Braber den, E.T., Ruijter de, J.E., Smits, H.T.J., Ginsel, L.A., Recum, A.F., Jansen, J.A., Effect of parallel surface microgrooves and surface energy on cell growth, *J Biomed Mater Res* **29**, 511 (1995).
- [18] Curtis, A., Wilkinson, C., Topographical control of cells, *Biomaterials* **18**, 1573 (1997).
- [19] USA, 5128170, (1992), Matsuda, T., Inoue, K., Kani, N..
- 10 [21] USA, 5330911, (1994), Hubbell, J.A., Massia, S.P., Desai, N.P..
- [20] USA, 5476509, (1995), Keogh, J.R..
- [23] USA, 5512474, (1996), Clapper, D.L., Hu, W.S..
- [22] USA, 5593814, (1997), Matsuda, T., Inoue, K., Tani, N..
- 15 [24] Yan M, Cai SX, Wybourne MN, Keana JFW. Photochemical functionalization of polymer surfaces and the production of biomolecule-carrying micrometer-scale structures by deep-UV lithography using 4-substituted perfluorophenyl azides. *J Am Chem Soc* **115**,;:814-816 (1993).
- [25] Vargo TG, Thompson PM, Gerenser LJ, Valentini RF, Aebischer P, Hook DJ, Gardella J. Monolayer chemical lithography and characterization of fluoropolymer films. *Langmuir* **8**, 130-134 (1992).
- 20 [26] Roberts C, Chen CS, Mrksich M, Martichonok V, Ingber DE, Whitesides GM. Using mixed self-assembled monolayers presenting RGD and (EG)3OH groups to characterize long-term attachment of bovine capillary endothelial cells to surfaces. *J Am Chem Soc* **120**, 6548-6555 (1998).
- 25 [27] Wollman EW, Kang D, Frisbie CD, Lorkovic IM, Wrighton MS. Photosensitive self-assembled monolayers on gold: photochemistry of surface-confined aryl azide and cyclopentadienylmanganese tricarbonyl. *J Am Chem Soc* **116**, 4395-4404 (1994).
- 30 [28] Amos RA, Guire PE, Surface modification of polymers by photochemical immobilization - a general method. 175. Scottsdale, Arizona, USA, Society of Biomaterials. 1-5-1991.

- [29] Garbassi, F., Morra, M., Occhiello, E. Modification techniques, in: *Polymer surfaces - from physics to technology*, Anonymous. John Wiley & Sons Ltd, Chapter 6-8, Chichester (England) 221ff. (1994).
- 5 [30] Ratner BD, Surface modification of polymeric biomaterials. 1. New York, Plenum Press (1997).
- [31] Ulbricht, M., Papra, A. Polyacrylonitrile enzyme ultrafiltration membranes prepared by adsorption, crosslinking, and covalent binding, *Enzyme Microb. Technol.* **20**, 61 (1997).
- 10 [32] Szleifer, I. Polymers and proteins: interactions at interfaces, *Current Opinion in Solid State & Materials Science* **2**, 337 (1997).
- [33] Sofia, S.J., Premnath, V., Merrill, E.W. Poly(ethylene oxide) grafted to silicon surfaces: grafting density and protein adsorption, *Macromolecules* **31**, 5059 (1998).
- 15 [33] Sofia, S.J., Premnath, V., Merrill, E.W. Poly(ethylene oxide) grafted to silicon surfaces: grafting density and protein adsorption, *Macromolecules* **31**, 5059 (1998).
- [34] Schröen, C.G.P.H., Cohen Stuart, M.A., Voort Maarschalk van der, K., Padt van der, A., Riet van't, K. Influence of preadsorbed block copolymers on protein adsorption: surface properties, layer thickness, and surface coverage, *Langmuir* **11**, 3068 (1995).
- 20 [35] Chan, C.M., Contact angle measurements, in: *Polymer surface modification and characterization*, 1<sup>st</sup> ed., Hanser Publishers, München, Chapter 2, 193ff. (1994).
- [36] Cuypers, P.A., Corsel, J.W., Janssen, M.P., Kop, J.M.M., Hermens, W.T., Hemker, H.C. The adsorption of prothrombin to phosphatidylserine multilayers quantitated by ellipsometry, *J Biol Chem* **258**, 2426 (1983).
- 25 [37] Azzam, R.M.A., Bashara, N.M. *Ellipsometry and polarized light*, 3<sup>rd</sup> ed., Elsevier Science, Amsterdam (1987).
- [38] Arnebrant, T., Ph.D.-thesis, Lund University, Lund, Sweden, (1987).
- 30 [39] Ulbricht, M., Hicke, H.G., Photomodifizierung von Ultrafiltrationsmembranen, I. Photochemische Modifizierung von Polyacrylnitril-Ultrafiltrationsmembranen mit Arylaziden, *Angew.Makromol.Chem.* **210**, 69 (1993).

- [40] Ulbricht, M., Hicke, H.G., Photomodifizierung von Ultrafiltrationsmembranen, II. Ultrafiltrationseigenschaften von mit Arylaziden modifizierten Polyacril-Ultrafiltrations Membranen, *Angew.Makromol.Chem.* **210**, 97 (1993).
- 5 [41] McCrackin, F.L., Passaglia, E., Stromberg, R.R., Steinberg, H.L., *J Res Nat Bur Stand Sect A* **67**, 363 (1963).
- [42] Barkholt, V., Jensen, A.L., Amino acid analysis: determination of cysteine plus half-cystine in proteins after hydrochloric acid hydrolysis with a disulfide compound as additive, *Analy.Biochem.* **177**, 318 (1989).
- 10 [43] Pethig, R. Dielectric and electronic properties of biological materials, New York, Wiley (1979).
- [44] Nygren H, Stenberg M. Immunochemistry at interfaces. *Immunology* **66**, 321-327 (1989).
- 15 [45] Chinn JA, Posso SE, Horbett TA, Ratner BD. Postadsorptive transitions in fibrinogen adsorbed to polyurethanes: changes in antibody binding and sodium dodecyl sulfate elutability. *J Biomed Mater Res* **26**, 757-778 (1992).
- [46] Rapoza RJ, Horbett TA. Postadsorptive transitions in fibrinogen: influence of polymer properties. *J Biomed Mater Res* **24**, 1263-1287 (1990).
- 20 [47] Nygren H. Real-time recording of antigen-antibody reactions at surfaces: interpretation of data and a statistical model. *Colloids Surf , B* **4**, 243-250 (1995).
- [48] Nygren H, Kaartinen M, Stenberg M. Determination by ellipsometry of the affinity of monoclonal antibodies. *J Immunol Methods* **92**, 219-225 (1986).